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MECHANISMS FOR ENDOCRINE DISRUPTING CHEMICAL ACTION ON SEXUAL DIFFERENTIATION OF THE RAT BRAIN

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MECHANISMS FOR ENDOCRINE DISRUPTING CHEMICAL ACTION ON SEXUAL DIFFERENTIATION OF THE RAT BRAIN

by

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Dedication

I dedicate this dissertation to the following people: to my husband, Brian R. Poplin, to my family and friends, and to my mentor, Dr. Andrea C. Gore. Their collective guidance, support, and faith in my abilities made this work possible.

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Endocrine disrupting chemicals (EDCs) are a class of environmental toxicants, of both natural and synthetic origin, that interfere with normal endocrine function. Exposure to EDCs during susceptible periods of development, particularly embryogenesis, can result in profound neurological and reproductive deficits. While the impact of developmental exposure to EDCs on reproductive function and behavior has been much studied, the underlying mechanisms responsible for these observed effects are not well understood. The goal of the research detailed in this dissertation is to elucidate the cellular and molecular targets by which a representative class of EDCs, polychlorinated biphenyls (PCBs), disrupts normal reproductive neuroendocrine function. My specific hypothesis is that PCBs cause changes in sexually dimorphic brain regions underlying sex-specific reproductive physiology and behavior through the perturbation of normal developmental apoptosis, with long-term consequences for reproductive success.

The studies detailed herein focus on three areas which contribute to an understanding of the effects of PCBs on neuroendocrine reproductive function: (1) the *in vitro* effects of PCBs on a neuroendocrine cell line, (2) developmental effects of PCBs on the gestationally exposed F1 generation, and (3) the physiological consequences of these developmental alterations for adult reproductive function. In the first section of this dissertation, the neurotoxic and endocrine disrupting effects of PCBs on a representative developing neuroendocrine cell model, the GT1-7 GnRH cell line, are investigated in time- and dose-response experiments. Treatment and dose-dependent effects are observed for GnRH peptide concentrations, cell viability, apoptotic and necrotic cell death, and caspase activation. In general, GnRH peptide levels are suppressed by high doses and longer durations of PCBs, and elevated at low doses and shorter time points. The suppression of GnRH peptide levels was partially reversed in cultures co-treated with the estrogen receptor antagonist ICI 182,780. All PCBs tested reduced viability and increased both apoptotic and necrotic cell death. The second section of this dissertation examines whether prenatal PCB exposure alters normal neuroendocrine development in the F1 generation, including sexual differentiation of the brain. Disruption of hypothalamic development is detectable as early as the day after birth (postnatal day (P) 1), as indicated by abnormal programmed cell death, and alterations in neuroendocrine gene and protein expression. The third section discusses the physiological impact of developmental PCB exposure on reproductive maturation and adult neuroendocrine function. Pubertal onset is advanced and estrous cyclicity irregular in PCB endocrine-disrupted females. Furthermore, sexual differentiation of female neuroendocrine systems is

masculinized/defeminized. Collectively, these results suggest that the disrupted sexual differentiation of the POA can be detected as early as the day after birth, effects that may underlie the adult reproductive phenotype.

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Nomenclature and Abbreviations

A	Aroclor
A1221	Aroclor 1221
A1254	Aroclor 1254
AGD	anogenital distance
AR	androgen receptor
ARC	arcuate nucleus of the hypothalamus
AVPV	anteroventral periventricular nucleus of the hypothalamus
BNST	bed nucleus of the stria terminalis
BW	body weight
cDNA	complementary deoxyribonucleic acid
DMSO	dimethyl sulfoxide
E	embryonic day
E2	estradiol
EB	estradiol benzoate
EDC	endocrine disrupting chemical
EIA	enzyme-linked immunoassay
EPA	Environmental Protection Agency
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
F	female
FSH	follicle stimulating hormone
GnRH	gonadotropin hormone releasing hormone
GSI	gonadosomatic index

HPG	hypothalamic pituitary gonadal
Inj	injection
IP	intraperitoneal
KISS-1	kisspeptin
LH	luteinizing hormone
M	male
MBH	mediobasal hypothalamus
MPN	medial preoptic nucleus
MPOA	medial preoptic area
mRNA	messenger RNA
NMDA	N-methyl-D-aspartic acid
NR2b	NMDA receptor subunit 2b
NR2c	NMDA receptor subunit 2c
P	postnatal day
P4	progesterone
PBDE	polybrominated diphenyl ether flame retardants
PCB	polychlorinated biphenyl
POA	preoptic area
PCB74	2,4,4',5-tetrachlorobiphenyl
PCB118	2',3,4,4',5' pentachlorobiphenyl
PCB138	2,2',3,4,4',5'-Hexachlorobiphenyl
PCB153	2,2',4,4',5,5'-Hexachlorobiphenyl
PCB180	2,2',3,4,4',5,5'- Heptachlorobiphenyl
POP	persistent organic pollutants
PR	progesterone receptor

RIA	radioimmunoassay
RM	reconstituted mixture
SC	subcutaneous
SDN-POA	sexually dimorphic nucleus of the preoptic area
SNB	spinal nucleus of the bulbocavernosis
T	testosterone
VMN	ventromedial nucleus

CHAPTER 1: GENERAL INTRODUCTION

The text in this section is modified from the book chapter "Reproductive Neuroendocrine Targets of Developmental Exposure to Endocrine Disruptors," Dickerson SM, Cunningham SL, and Gore AC, in Endocrine Disruptors and Puberty (2010), with permission from the publisher.

HYPOTHALAMIC CONTROL OF REPRODUCTION

Endocrine disruptors (EDCs) are chemicals in our environment that interfere with the normal functioning of an organism's endocrine systems, including those involved in reproduction. In vertebrate species, reproductive function is regulated by the hypothalamic-pituitary-gonadal (HPG) axis, which includes the gonadotropin-releasing hormone (GnRH), pituitary gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)], and gonadal steroid hormones. The hypothalamus, located at the base of the brain, maintains homeostasis of a variety of physiologic functions by acting as a central processor, integrating converging inputs from sensory and autonomic systems. Importantly, the hypothalamus coordinates cues related to the organism's internal and external environment with the timing of reproductive capacity, in order to ensure that the energetically costly process of reproduction occurs only during the most favorable conditions. Although the hypothalamic GnRH neurons provide the primary driving force onto other HPG levels, all three levels must function properly for the appropriate control of reproduction.

The GnRH neuroendocrine cells controlling reproduction have their neural somata (cell bodies) in the hypothalamus, and extend an axon to the base of the hypothalamus, the median eminence. For the control of HPG processes, GnRH-releasing cells respond to changing environmental conditions by rapidly adjusting their release of the GnRH peptide into the portal capillary vasculature that transports the peptide to the anterior pituitary gland. From the pituitary, LH and FSH are released into the general circulatory system, and target the gonads to drive steroidogenesis and gametogenesis. Gonadal steroids then act upon steroid hormone receptors, primarily estrogen receptors (ERs), androgen receptors (ARs) and progesterone receptors (PRs) that are widely and heterogeneously expressed in target tissues, both reproductive and non-reproductive, including the reproductive tract and genitalia, breast, fat, bone, muscle, kidney, liver, and many other organ systems. Of relevance to the regulation of HPG function, steroid hormone receptors are also expressed in the central nervous system (CNS) and pituitary gland. These receptors enable steroid feedback to be exerted on the hypothalamic-pituitary levels of the HPG axis.

While GnRH neurons in the hypothalamus control pituitary and gonadal hormone release through feedforward regulatory mechanisms, and in turn are regulated by gonadal steroid hormones through feedback regulatory mechanisms, it is important to note that GnRH neurons themselves do not express most of the nuclear sex steroid hormone receptors. This raises the question: how are GnRH neurons regulated by steroid feedback? The answer is provided by observations that the CNS, and particularly the hypothalamus is abundant in ERs, ARs and PRs (Chakraborty and Gore, 2004). A

network of these steroid-sensitive neurons in the hypothalamus converges upon the GnRH cells. This neural circuitry is quite heterogeneous, as the neurons can arise from a variety of locations, and they can release a diversity of neurotransmitters. This circuit may seem unduly complex, but it makes sense in considering that reproduction must be properly coordinated with the internal and external environment. Many CNS pathways are needed to convey all of the specific types of information to the GnRH system, which is the final common pathway to the pituitary gland.

Sex differences in HPG function

There are important sex differences in the control of reproductive physiology and behavior. Although both male and female mammals release GnRH in a pulsatile manner, at intervals of about 30-120 minutes depending upon species, the GnRH neurosecretory system differs between males and females in a fundamental way. In females, the amplitude and frequency of GnRH release varies profoundly across the reproductive cycle, whereas in males, GnRH is fairly consistently released in pulses. Moreover, feedback of gonadal steroid hormones onto GnRH cells is always negative in males, whereas it shifts from negative to positive during the preovulatory surge in females. CNS inputs to GnRH neurons are also sexually dimorphic in their regulation of GnRH release and in their neuroanatomical properties. For example, important inputs to GnRH cells arise from the anteroventral periventricular nucleus (AVPV), which regulates GnRH release via expression of ERs (Petersen and Barraclough, 1989; Wintermantel *et al*.,

2006), and is approximately two times larger in volume and cell number/density in adult females than in males (Bleier *et al*., 1982; Sumida *et al*., 1993). The preovulatory GnRH/gonadotropin surge in females is regulated by inputs from the AVPV to GnRH neurons, a process that does not occur in males. Conversely, the sexually dimorphic nucleus of the preoptic area (SDN-POA), the size of which is correlated with regulation of sexual behavior and levels of gonadotropins, has a volume roughly two to four times higher in male rats than in females (Gorski *et al*., 1978; Gorski *et al*., 1980). Normal reproductive function depends on proper development of these and other sexually dimorphic neural circuits that take shape during the process of brain sexual differentiation, which in rodents occurs during late embryonic/early postnatal development (Barraclough, 1961).

The concept of a critical period of brain sexual differentiation has been proposed and studied by neuroendocrinologists for decades. However, its importance to environmental endocrine disruption is only just recently becoming clear. A growing body of scientific evidence has illuminated the vulnerability of the developing neonate to exposure to environmental EDCs that may disrupt sexual differentiation, but effects of which may not be manifested until much later in life. The importance of developmental exposures is a focus of the current article, and will be elaborated upon below.

Sexual differentiation of the hypothalamus: Hormones and apoptosis

In vertebrate species, sex differences in reproductive physiology and behavior are attributed to phenotypic differences in the neuroanatomy and neurochemistry of specific brain regions. These differences become organized during critical developmental windows, particularly the late embryonic and early postnatal periods, and are permanent. At these times, sex differences in levels of gonadal hormones are large, and contribute to morphological and functional differences in the size, cell number, phenotype, and neurochemistry of hypothalamic brain regions (Gorski *et al*., 1980; Simerly *et al*., 1984; Simerly *et al*., 1985; De Vries and al-Shamma, 1990; Sumida *et al*., 1993). A key component in the shaping of the brain into a male or female typical pattern is the presence of the testicular hormone testosterone, and its aromatization to estradiol. Neonatal female rodents have relatively low levels of gonadal hormones because the perinatal ovary is quiescent, and the steroid binding protein alpha (α)-fetoprotein forms a complex with any circulating endogenous estrogen, which prevents it from entering the brain. Conversely, around the time of birth the testis releases a surge of testosterone in neonatal males, which is not bound by α-fetoprotein (Bakker and Baum, 2008). Once it enters the brain, testosterone may act directly upon androgen receptors (ARs), or may it may be converted to estradiol, the endogenous ligand for estrogen receptors (ERs), by the enzyme aromatase. Consequently, while the female brain develops under relatively low levels of steroid hormones, the male brain is exposed to high levels of both testosterone and estradiol. Thus, testosterone and estradiol act directly upon their cognate receptors in a sexually dimorphic manner to organize the brain into a sex-appropriate pattern. Not

surprisingly, alterations in the normal hormonal milieu during this phase of development can have deleterious effects, which may manifest as deficits in reproductive function and behavior during adulthood.

Apoptosis, an essential component of normal neuronal development, is one of the mechanisms through which hormones sculpt the brain during sexual differentiation (Forger *et al*., 2004). Depending upon the hypothalamic nucleus, estradiol may stimulate or inhibit apoptosis through the interaction of ligand bound ER dimers with nuclear response elements that promote cell survival or death. For instance, the number of apoptotic cells in the medial preoptic nucleus, spinal nucleus of the bulbocavernosis (SNB) (Nordeen *et al*., 1985), bed nucleus of the stria terminalis (BNST) (Chung *et al*., 2000), and SDN-POA (Davis *et al*., 1996a) is higher in female rodents than in males during early postnatal development. In males, these regions are involved in penile erection, and in masculine sexual physiology and behavior. On the other hand, the number of apoptotic cells in the anteroventral periventricular nucleus (AVPV) is greater in male rodents compared to females during a similar time period (Sumida *et al*., 1993; Yoshida *et al*., 2000), and this region is accordingly larger in females than in males.

There are discrete hormone-sensitive time periods during which the volumes of these particular regions are vulnerable to substances that act on steroid hormone receptors (Rhees *et al*., 1990a; Rhees *et al*., 1990b). For example, exposure of developing female rats to testosterone or excessive levels of estradiol (more than can be bound by circulating α-fetoprotein) results in a larger, masculinized SDN-POA in adulthood, while castration of neonatal males results in a smaller, feminized SDN-POA (Rhees *et al*., 1990a).

Similarly, the rat AVPV is sensitive to hormone influence during a period spanning late prenatal and early postnatal development (Patisaul *et al*., 2007). Orchidectomy of neonatal males and treatment of neonatal females with testosterone or estradiol abolishes the sex difference in AVPV volume and cell number (Simerly *et al*., 1985; Simerly, 1989; Sumida *et al*., 1993; Davis *et al*., 1996b). As will be discussed in greater detail below, perinatal exposure to environmental EDCs, which do not bind to the protective α-fetoprotein, also causes similar changes in the volume of sexually dimorphic nuclei (Masutomi *et al*., 2003; Ikeda *et al*., 2005; Yamamoto *et al*., 2005b).

Sexual dimorphism is not limited solely to anatomical differences or number of cells in these brain nuclei; there are also marked differences in the phenotype of cells in sexually dimorphic brain regions. For instance, the AVPV of female rodents expresses a greater number of cells that are immunopositive for tyrosine hydroxylase (Simerly *et al*., 1997; Forger *et al*., 2004), kisspeptin (Simerly, 2002; Clarkson and Herbison, 2006; Kauffman *et al*., 2007a; Bateman and Patisaul, 2008; Gonzalez-Martinez *et al*., 2008), and estrogen receptor (ER) (Simerly *et al*., 1997; Orikasa *et al*., 2002). In the end, adult sex-specific reproductive behaviors are the manifestation of proper organization of the brain by sex steroid hormones during perinatal development. Thus, late prenatal and early postnatal development comprises a critical window during which the functional morphology and neurochemistry of these sexually dimorphic regions are established. Exposure to endocrine active exogenous substances or even to maternal or endogenous hormones at the inappropriate time could cause permanent alterations in adult reproductive function. To follow is a summary of the literature investigating the effects of

endocrine disrupting chemicals on neonatal development of reproductive neuroendocrine function. For more detailed information regarding compounds, age and duration of treatment, dosage, exposure route, and species, refer to **Tables 1-3**.

ENDOCRINE DISRUPTION OF NEUROENDOCRINE SYSTEMS

Environmental EDCs were originally defined by the United States Environmental Protection Agency (EPA) as "exogenous agents that interfere with the synthesis, secretion, transport, binding action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or, behavior" (Kavlock *et al*., 1996). The EPA has gone on to expand its definition to: "An exogenous substance that changes endocrine function and causes effects at the level of the organism, its progeny, and/or (sub)populations of organisms." Regardless of which definition one uses, it is clear that EDCs have a diversity of targets and act through a number of pathways related to endocrine control of homeostasis. In addition to their effects on the HPG axis, EDCs may also have actions on other neuroendocrine hormonal systems, including the thyroid (hypothalamic-pituitary-thyroid axis), stress (hypothalamic-pituitary-adrenal axis), growth (somatotropic axis), lactotrophic axis, neuropeptides, and other metabolic hormones. The current chapter will primarily focus on the effects of developmental EDC exposure on reproductive neuroendocrine targets in the perinatal and pubertal mammal. Regarding the effects of developmental EDC exposure on other hormonal systems, refer to the following reviews on the thyroid axis

(Zoeller, 2005; Zoeller, 2007; Anderson, 2010); glucocorticoids (Schantz and Widholm, 2001; Desvergne *et al*., 2009); growth (Scarth, 2006); and general metabolism (Swedenborg *et al*., 2009). Endocrine disruption is also a significant concern for non-mammalian species. Invertebrates and aquatic species may be particularly vulnerable, as they have a large surface-to-volume ratio and lack a barrier such as skin to protect against environmental toxicants, and they are swimming in the very substance (water) in which many EDCs are dissolved or in solution. Egg-laying animals also have vulnerability through exposure of the eggshell to the environment. Although this chapter focuses on effects of EDCs on the HPG axis of mammals, we refer readers to the following articles and reviews on other species/classes: nematodes (Hoss and Weltje, 2007); Drosophila (Hirsch *et al*., 2010); aquatic invertebrates (McLachlan *et al*., 2001; Hotchkiss *et al*., 2008; Kloas *et al*., 2009); fish (Rempel and Schlenk, 2008); reptiles (Sheehan *et al*., 1999; Guillette and Gunderson, 2001); and birds (Panzica *et al*., 2007).

As will be discussed in greater detail, the mediation of the endocrine disrupting effects of EDCs on neuroendocrine reproductive function often occurs through (but is not limited to) steroid hormone receptors, particularly ERs and ARs, which are expressed abundantly in the hypothalamus, but are differentially affected by EDCs. Some effects may also occur directly upon GnRH neurons, as illustrated by the section on GnRH cell lines. Later in this chapter, we will highlight the evidence for neuroendocrine effects of one of the most extensively studied classes of EDCs: the polychlorinated biphenyls (PCBs). Although they will not be discussed herein, several other classes of EDCs are

also known to disrupt the HPG axis, including phytoestrogens, industrial organohalogen compounds, pesticides, bisphenol A, and phthalates (**Figure 1.1).**

A PROTOTYPICAL EDC: POLYCHLORINATED BIPHENYLS

Regardless of whether they are naturally occurring or man-made, EDCs are chemicals in our environment that act through numerous mechanisms to block, imitate or otherwise modify normal hormonal function. These alterations to endogenous endocrine activity may involve the direct or indirect interaction of EDCs with steroid hormone or neurotransmitter receptors, alterations in levels of bioavailable endogenous hormones or their receptors by EDCs, or actions on enzymes involved in the biosynthesis or degradation of endogenous hormones, among other potential mechanisms. From a historical perspective, the first endocrine disruptors to be identified were estrogenic in nature, although it is now known that EDCs may also be anti-estrogenic or anti-androgenic in nature. Hundreds of chemicals have already been identified as known EDCs, and systematic screening by the Endocrine Disruptor Screening Program (EDSP) by the EPA of pesticides and other environmental contaminants is currently underway. This section will highlight one class of EDCs known to target reproductive neuroendocrine systems: polychlorinated biphenyls (PCBs), structures of which are shown in **Figure 1.1**. In this section, we provide background information about this class of EDC, and in subsequent sections, we review evidence for their actions on

neuroendocrine systems, particularly when exposure occurs during critical developmental windows such as the prenatal, early postnatal, and pubertal periods.

Figure 1.1: Chemical Structure of Representative EDCs

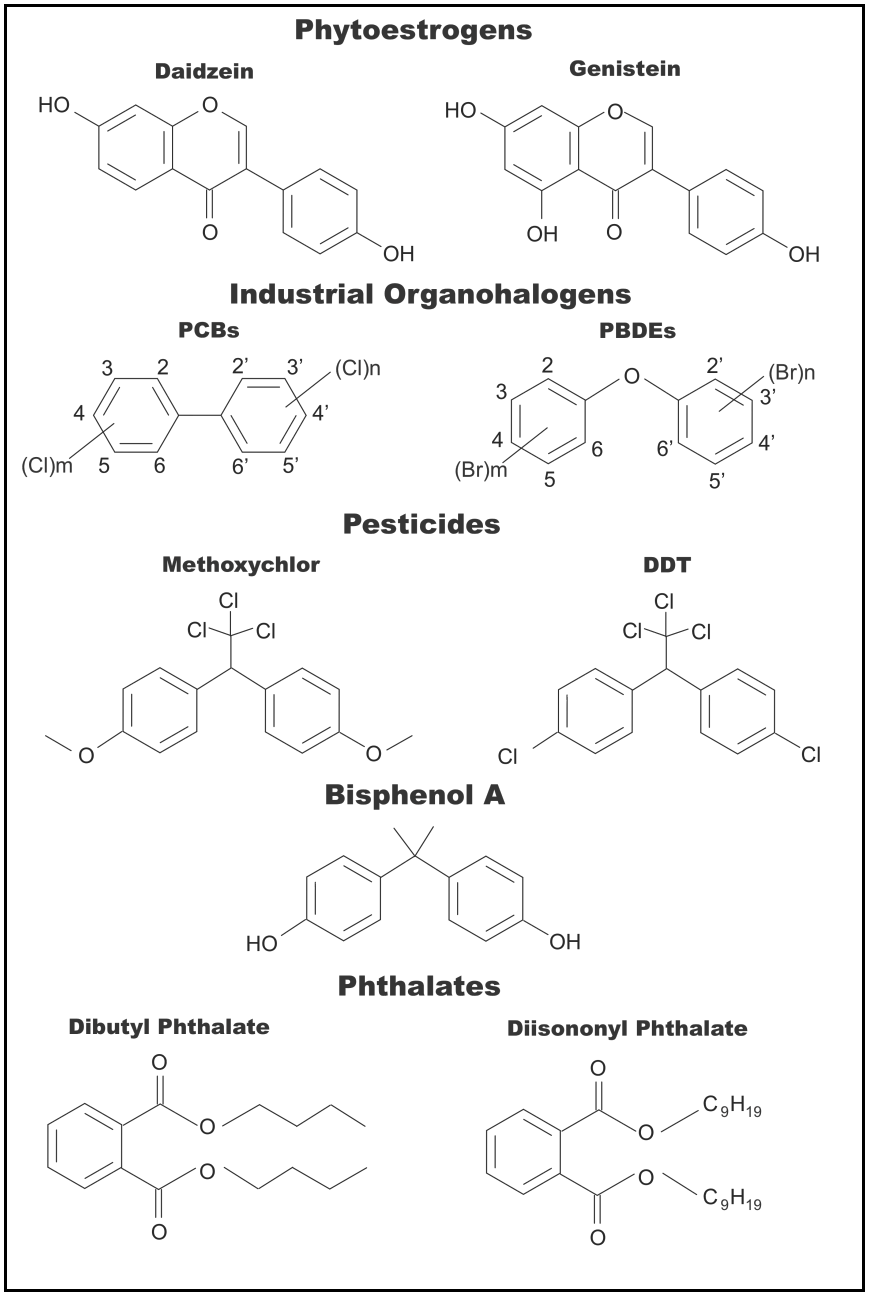


Figure 1.1. Structures of representative endocrine disruptors.

Chemical Properties of PCBs:

Industrial organohalogens encompass an array of compounds used for or produced as a byproduct of industrial applications. They include a diverse group of chemicals such as polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), polybrominated diphenyl ether flame-retardants (PBDEs), chlorinated paraffins, furans, and dioxins. As a whole, members of this family of persistent organic pollutants (POPs) are generally structurally stable and resistant to environmental or biodegradation, and are routinely detected in the tissues of humans and wildlife.

Much of the information regarding the effects of industrial organohalogens on the reproductive axis comes from studies involving PCBs (**Figure 1.1**). PCBs are a group of related compounds that consist of chlorine atoms arranged around a biphenyl core, a feature that contributes to their ability to interact with various hormone and neurotransmitter receptors as agonists, antagonists, or with mixed activity. Endogenous estradiol has a phenolic moiety in its A ring, and the interaction of this moiety with ERs in particular is mimicked by environmental EDCs such as PCBs. Although widely used between the 1930s and 1970's, manufacture of PCBs was prohibited in 1977 due to their toxicity. Ironically, the same qualities that made use of PCBs desirable for such industrial applications as capacitors, transformer oil, sealants, paints, etc., contributed to their persistence in the environment and their toxicity. For instance, PCBs resist degradation, and because they bioaccumulate and biomagnify, they are still prevalent as complex mixtures throughout the food chain and in environmental samples. Remarkably, they

contaminate even remote areas, such as the Arctic and Antarctic regions (Jenssen, 2006; Letcher *et al*., 2009), due to movement of marine biota associated with fluctuating climate, as well as through air and water currents. Exposure of humans and wildlife to PCBs occurs mainly through consumption of contaminated food, and has been linked to reproductive deficits. For example, farmed salmon has relatively high concentrations of PCBs (among other EDCs) (Hites *et al*., 2004) and pregnant women have been advised to limit consumption of this otherwise healthy food choice to once monthly.

Based on their chemical structure, PCBs are grouped into one of the following three categories: coplanar, dioxin-like coplanar, or non-coplanar. In addition, the structural properties of each PCB class determine whether and how (i.e. agonist, antagonist) they interact with hormone and neurotransmitter receptors. Along with actions on ERs, PCBs target a broad range of hormone receptors (thyroid hormone receptor, androgen receptor), neurotransmitter receptors (e.g. acetylcholine, dopamine, GABA, and serotonin), hormone-binding proteins (e.g. thyroid hormone binding protein), as well as orphan receptors (e.g. aryl hydrocarbon receptor) and intracellular signaling receptors (e.g. ryanodine receptor). In addition, *in vitro* studies have shown that the hydroxylated metabolites of PCBs inhibit estradiol sulfotransferase, which may prolong the bioavailability of endogenous estradiol (Kester *et al*., 2000). Furthermore, depending on their structure, individual PCBs may be estrogenic, anti-estrogenic, or anti-androgenic. To complicate matters, because PCBs are present in human and wildlife samples as mixtures of the most persistent congeners and their hydroxylated metabolites, additive, synergistic, or antagonistic effects between PCBs may occur (Bergeron *et al*., 1994).

EDC EFFECTS ON THE GNRH SYSTEM AND DEVELOPING HYPOTHALAMUS

EDC Effects on GnRH Cell Lines

GnRH neurons in the hypothalamus are notoriously difficult to study. Their location at the base of the brain makes them inaccessible to traditional electrophysiological measures, as does the fact that they are scattered across a continuum in the preoptic area of the hypothalamus. These have been significant obstacles to research in the hypothalamic control of HPG function. The GnRH GT1 cell lines, created in the late 1980's (Mellon *et al*., 1990) have proven to be valuable resources for understanding GnRH properties. They have many neural properties, and they synthesize and release the GnRH peptide in a pulsatile manner (Martinez de la Escalera and Clapp, 2001). To date, this cell line has been used to study effects of the EDCs, PCBs, dioxin (TCDD) and organochlorine (methoxychlor, chlorpyrifos) and pyrethroid (tefluthrin) pesticides, and the phytoestrogen coumestrol. A brief summary of those results is provided, with evidence that most of these compounds directly affect these immortalized GnRH cells (**Table 1**).

Table 1. Effects of environmental endocrine disruptors on GnRH GT1 cell lines.

EDC treatment	GT1 cell morphology/viability	GnRH peptide concentrations	GnRH gene expression	Reference
PCB (Aroclor 1221) for 24 hrs @ 0.01-100 μM	Stimulate neurite outgrowth and proliferation	Stimulate at 10 μM dose	Stimulate at 1 μM (no effect higher/lower doses)	(Gore, 2002; Gore *et al*., 2002)
PCB (Aroclor 1254) for 24 hrs @ 0.01-100 μM	Cause some cell death; retraction of neural processes	No effect	Stimulate at low (0.01 μM) doses (no effect higher doses)	(Gore *et al*., 2002)
Chlorpyrifos for 24 hrs @ 0.01-100 μM	Neurite extension and increased confluency	No effect	Stimulate at 0.01-1 μM; inhibit at 1-100 μM	(Gore, 2002)
Methoxychlor for 24 hrs @ 0.01-100 μM	Mild neurotoxicity	No effect	Stimulate at 0.01-1 μM; inhibit at 1-100 μM	(Gore, 2002)
TCDD (1-100 nM) for 1-72 hrs	No effect on GT1-7 cell number	No effect on GnRH peptide concentrations	No effect on GnRH promoter-reporter expression in transfected cells	(Petroff *et al*., 2003)
Coumestrol (1 nM-10 μM) for 6-48 hr	N.S.	N.S.	Coumestrol (1 μM) inhibits GnRH mRNA; greatest effect of 1 μM @ 6 hr	(Bowe *et al*., 2003)

N.S. = not studied

The first studies to investigate actions of EDCs on GT1-7 cells were those from our laboratory, investigating effects of PCBs and organochlorine pesticides. Dose-response analysis of the PCB mixtures, Aroclor 1221 and Aroclor 1254, for 24 hours demonstrated differential effects on cell morphology, GnRH release, and GnRH gene expression (Gore, 2002). Specifically, GnRH peptide release was stimulated by Aroclor 1221 (but not Aroclor 1254), an effect that was blocked by the estrogen receptor antagonist, ICI 182780. GnRH mRNA levels were elevated at the intermediate concentration of 1 μM but were unchanged at higher/lower concentrations. By contrast, Aroclor 1254 stimulated GnRH mRNA levels only at the lowest concentration (0.01 μM). Finally, GT1-7 cell morphology was altered by PCB treatment, with Aroclor 1221 increasing cell confluence and promoting neurite outgrowth. Aroclor 1254 tended to cause some cell death and for GT1-7 cells to retract their neural processes. As a whole, these data support the effects of PCB mixtures on this immortalized cell line.

Effects of the pesticides, chlorpyros and methoxychlor, have been studied on GnRH peptide and mRNA levels in GT1-7 cells (Gore, 2002). Neither pesticide affected GnRH peptide concentrations. Chlorpyrifos increased confluency of the cells and stimulated neurite extension, whereas methoxychlor was mildly neurotoxic. Effects of the pesticides on GnRH gene expression were similar: both stimulated GnRH mRNA levels at lower concentrations, and inhibited levels at the highest concentrations.

Other laboratories have investigated actions of other EDCs on more limited endpoints in GT1 cells. Petroff *et al*. (2003; **Table 1**) showed that the dioxin TCDD had no effect on GnRH secretion, on promoter-reporter expression in transfected GT1-7 cells,

or on GT1-7 cell number (Petroff *et al*., 2003). They also demonstrated that their GT1-7 cells did not express the aryl hydrocarbon receptor, possibly explaining the lack of effect of dioxin on this orphan receptor.

The effects of the pyrethroid insecticide tefluthrin were tested on ion currents in GT1-7 cells (Wu *et al*., 2009b). Cells were treated with this compound at 10 μM, and currents were measured. Current amplitude and firing frequency were both increased by tefluthrin application. It will be interesting to examine other effects of this pyrethroid, including GnRH peptide release and gene expression.

Finally, coumestrol has been studied for its effects on GnRH gene expression in GT1-7 cells, with a focus on the mediation of these actions by the ERβ (Bowe *et al*., 2003). In general, coumestrol had inhibitory effects on GnRH mRNA levels, with the greatest effect seen at the 6-hour time point and the 1 μM concentration (**Table 1**). A role for ERβ was established through use of the specific antagonist, *R,R*-diethyl tetrahydrochrysene (*R,R*-THC), which blocked the inhibitory effect of coumestrol on GnRH mRNA levels (Bowe *et al*., 2003). These data support the actions of this phytoestrogen directly on GnRH immortalized neurons through this ER subtype.

As a whole, these studies on the GT1-7 cell line support the likelihood that environmental EDCs can directly target GnRH neurons. Although it is critical to extend these studies to *in vivo* models, they provide a potential mechanism for effects of these compounds on HPG systems.

PCB Effects on the Developing Hypothalamus

Brain development is a highly complex and coordinated sequence of events that begins early on during embryogenesis and continues through puberty and into early adulthood. The perinatal brain is particularly vulnerable to disruption by EDCs, as it is during this time that proper development of the central nervous system and neuroendocrine reproductive axis is fine-tuned by factors that are influenced by endocrine hormones, including genes, neurotransmitters, and growth/survival factors (Dickerson and Gore, 2007; Gore, 2008b). Importantly, through the developmental process of brain sexual differentiation, the organism acquires sex-appropriate brain morphology and function. As will be discussed in greater detail below, disruption of any of these development processes may permanently alter brain morphology and function, causing disruptions in adult reproductive physiology and behavior later in life. This section will highlight evidence that early exposures to PCBs may disrupt this crucial process.

BRAIN SEXUAL DIFFERENTIATION

As mentioned earlier, neonatal exposure of developing humans and animals to organohalogen compounds is a global public health and environmental concern, as humans and wildlife even from remote areas have appreciable levels of this class of ED in their body burden. Early studies with pregnant laboratory animals showed that PCBs readily cross the placenta, and are also passed to the suckling young (Masuda *et al*.,

1978a; Masuda *et al*., 1978b; Ando *et al*., 1985). Trans-placental transfer of PCBs has been confirmed in humans, as levels in the serum of newborns corresponds with maternal levels (Lackmann *et al*., 1999; Covaci *et al*., 2002; Park *et al*., 2008). Moreover, since they are small, lipophilic compounds, PCBs also cross the blood-brain-barrier, and have been detected in human fetal brain tissue at an average concentration of 50 ppb (Lanting *et al*., 1998). Age at parity may be an important consideration, as presumably the body burden of organohalogens increases with age, and many women are delaying childbirth in industrialized nations. Indeed, epidemiological evidence suggests that children born to older mothers may have increased exposure to compounds such as PCBs (Lackmann *et al*., 1999). This type of exposure may have relevance for human health, as a growing body of evidence shows that developmental exposure to organohalogens such as PCBs have effects on the developing neuroendocrine system of laboratory animals (**Table 2**).

The effects of neonatal exposure to PCBs on brain sexual differentiation has been studied in several animal models, and collectively these data suggest that this endpoint is sensitive to disruption by these environmental pollutants. Several studies have reported that such exposure to PCBs alters expression of nuclear hormone receptors and aromatase activity, an effect that seems to be dependent upon the specific PCB molecule(s). For instance, Hany *et al*. (1999) found that newborn male pups gestationally exposed to a reconstituted mixture of PCBs formulated after the congener pattern found in human milk exhibited significantly reduced aromatase activity in the hypothalamus/preoptic area, while no effect was observed in pups similarly exposed to Aroclor 1254 (Hany *et al*., 1999). A study by Colciago *et al*. (2006) found that prenatal Aroclor 1254 (A1254)

exposure during late gestation had no effect on hypothalamic aromatase expression or activity (Colciago *et al*., 2006). Hypothalamic androgen receptor protein expression is sensitive to even short-term prenatal PCB exposure, as acute A1254 exposure during embryonic development reduced hypothalamic AR protein expression in embryonic female rats at embryonic day 20 in rats, which is late in gestation. In a follow-up study by the same group, Colciago *et al*. (2009) found that perinatal exposure to a reconstituted PCB mixture caused an increase in hypothalamic aromatase expression in male rats at weaning, while females were unaffected (Colciago *et al*., 2009). In contrast, only female rats had decreased levels of hypothalamic 5-alpha-reductase, the enzyme that converts testosterone into the more potent androgen dihydrotestosterone, at the time of weaning. Whether these reductions in aromatase and 5-alpha reductase activities result in reduced androgen action in the developing female brain or impairments on adult female sexual behavior are not clear. In a related study using the same experimental design, Pravettoni et al. (2005) evaluated the effect of PCBs on the developmental profile of aryl hydrocarbon receptors (AhR), a nuclear orphan receptor that mediates effects of dioxins and dioxin-like PCBs, in the male and female rat hypothalamus. The group found that AhR gene expression progressively increases in the male hypothalamus during embryonic development, and that A1254 further stimulated gene expression of AhR in males. No effect was observed in females (Pravettoni *et al*., 2005).

Estrogen receptor, and estrogen-dependent gene expression is also affected by developmental exposure to PCBs. For instance, Lichtensteiger *et al*. (2003) found that gestational exposure to A1254 increased ERα gene expression in the ventromedial

nucleus of the hypothalamus (VMN), a region important for feminine sexual behavior, in female rat embryos (Lichtensteiger, 2003). In contrast, progesterone receptor gene expression in the female VMN was decreased by treatment in the same study. Together, the studies highlighted above suggest that gestational exposure to PCBs affects expression of nuclear receptors in sexually dimorphic brain regions. However, the impact of these alterations in gene and protein expression on the volume of these sexually dimorphic nuclei is not yet clear.

In a recent study by Shimada *et al*. (2009) investigating the toxicogenomics of prenatal exposure to A1254 in male mice at birth, the gene expression of α-fetoprotein in whole brain extracts was increased several-fold (~6.8-fold change) compared to control (Shimada *et al*., 2009). Since PCBs do not bind α-fetoprotein, this could have implications for the bioavailability of endogenous estradiol aromatized from fetal testis. As a whole, these studies suggest acute disruption of hormone receptors and metabolism/biosynthesis of hormones by organohalogen EDCs.

SERUM HORMONES AND ANOGENITAL DISTANCE

In addition to disruptions in the normal pattern of gene and protein expression in the developing brain, early exposure to PCBs also alters serum hormone levels and anogenital distance (AGD) in exposed offspring. For example, Kaya *et al*. (2002) reported decreased serum estradiol and testosterone in female rats exposed to a reconstituted mixture of PCBs throughout the period of gestation and lactation, while only serum testosterone was reduced in exposed males (Kaya *et al*., 2002). The effect of PCBs on serum hormones may be dependent upon the composition of the congeners and the dose used. For instance, gestational exposure to low doses (3–30 μg/kg/day) of the

individual PCB congeners PCB126 or PCB169 resulted in reduced levels of serum testosterone in weanling males (Yamamoto *et al*., 2005a). In contrast, another group showed that when rats were exposed to a higher doses (4 mg/kg/day) throughout the period of late gestation until weaning, PCB126 combined with PCB138, PCB153, and PCB180 had no effect on testosterone in exposed males (Cocchi *et al*., 2009). Finally, Lilienthal *et al*. (2006) found that gestational exposure to A1254 caused reductions in serum estradiol in weanling male rats, while female estradiol levels were not affected. As a whole, these data support effects of PCBs on hormone levels in early life (Lilienthal *et al*., 2006).

PCB effects on neonatal AGD are dependent upon compound and sex. For instance, a study investigating the effects of gestational and lactational exposure to the dioxin-like coplanar congener PCB118 found that male rats had hypermasculinized AGD throughout early postnatal development, although the effects on female AGD were not reported (Kuriyama and Chahoud, 2004). Interestingly, A1254 exposure hyperfeminized (shortened) the AGD in female pups, while male AGDs were unchanged compared to control (Lilienthal *et al*., 2006). The effect of A1254 on female AGD seems to be dose-dependent, as gestational exposure to A1254 over the same time period with a lower dose caused an increase in the AGD of treated females, compared to control females (Ceccatelli *et al*., 2006). However, the same study found that similar doses of PBDE-99, a major congener found in human breast milk samples, had no effect on AGD in treated females. Similarly, exposure to the non-coplanar congener PCB47 and the dioxin-like

congener PCB77 during gestation defeminized AGD in female neonates, while males were unaffected (Wang *et al*., 2002).

Collectively, the studies mentioned herein demonstrate the sensitivity of sex steroid hormone-dependent gene expression in the brain, anogenital distance, and serum steroid hormone levels in the neonate to developmental PCB exposure. More studies need to be conducted, in order to understand the impact of developmental PCB exposure on sexually dimorphic brain regions in exposed neonates.

Table 2: Effects of PCB Exposure on Neonatal Neuroendocrine Development

Effect	*Sex*	*Compound*	*Age at Treatment*	*Method of Exposure*	*Age at Testing*	*Organism*	*Reference*
Neuroendocrine Gene/Protein Expression							
↓ Androgen Receptor protein expression-hypothalamus	F	A1254	E15-E19	Maternal gavage (25 mg/kg)	E20	Rat	Colciago *et al*., 2006
↓ Aromatase activity	M	PCBs (RM)	E0-P0	Maternal diet (4 mg/kg bw)	P0	Rat	Hany *et al*., 1999
↑ aromatase expression No Δ: aromatase expression, ↓ 5 α-reductase-1	M F	PCBs (RM)	E15-P21	SC inj (10 mg/kg/day from E15-E19, then twice weekly thereafter)	P21	Rat	Colciago *et al*., 2009
↑ Aryl hydrocarbon receptor gene expression-hypothalamus	M	A1254	E15-E19	Maternal gavage (25 mg/kg)	E20	Rat	Pravettoni *et al*., 2005
↑ ERα, No Δ ERβ gene expression-VMN,↓ Progesterone receptor gene expression-VMN	F	A1254	E10-E18	SC inj (10 mg/kg bw/day)	E18	Rat	Lichtensteiger *et al*., 2003
↑ α-fetoprotein gene expression in brain	M	A1254	E5 – P1	Maternal gavage (18 mg/kg every third day)	P1	Mice	Shimada *et al*., 2009
Hormones and Anogenital Distance							
↓ Serum estradiol and ↓ testosterone ↓ Serum testosterone	F M	PCBs (RM)	E0-P21	Maternal diet (2, 4 mg/kg bw)	P21	Rat	Kaya *et al*., 2002
↓ Serum	M	PCB126	E7-E21	Maternal	P21	Rat	Yamamoto *et*

testosterone		PCB169		gavage (3, 30 µg/kg/day)			*al*., 2005
↓ Serum estradiol ↓ Anogenital distance	M F	A1254	E10-E18	SC inj (30 mg/kg bw)	P21	Rat	Lilienthal *et al*., 2006
↑ Anogenital distance	M	PCB118	E6	Maternal gavage (375 µg/kg)	P3, P15, P21	Rat	Kuriyama *et al*., 2004
↑ Anogenital distance	F	A1254	E10-E18	SC inj (10 mg/kg/bw)	P2	Rat	Ceccatelli *et al*., 2006
↑ Anogenital distance	F	PCB47 PCB77	E7-E18	IP inj (20 mg/kg bw); (0.25 mg, 1 mg/kg bw)	P1	Rat	Wang *et al*., 2002

Abbreviations:

↑: increased; ↓: decreased; A1221: Aroclor 1221; A1254: Aroclor 1254; AGD: anogenital distance; AVPV: anteroventral periventricular nucleus of the hypothalamus; bw: Body weight; E: embryonic day; ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; F: female; GnRH: gonadotropin hormone releasing hormone; Inj: injection; IP: Intraperitoneal; M: male; MPOA: medial preoptic area; MPN: medial preoptic nucleus; mRNA: messenger RNA; No Δ: unchanged; P: postnatal day; P4: progesterone; PCB: polychlorinated biphenyl; PR: progesterone receptor; RM: reconstituted mixture; SC: subcutaneous; SDN-POA: sexually dimorphic nucleus of the preoptic area; VMN: ventromedial nucleus of the hypothalamus.

PCB Effects On The Pubertal Transition

Puberty is the developmental process whereby the juvenile organism reaches adulthood, and is initiated by the activation of the HPG axis. This protracted transitional period is marked by acceleration of growth, development of secondary sexual characteristics, and culminates with the attainment of adult reproductive function. Following organization of the brain throughout early postnatal development, during which the HPG axis is active, a quiescent phase characterized by low levels of gonadotropins and circulating steroid hormones maintains inhibitory control over HPG activity. During the pubertal transition to adult reproductive capacity, reactivation of the GnRH neurosecretory system is manifested as increased pulse frequency and amplitude of hypothalamic GnRH release. This in turn, promotes pulsatile release of the gonadotropins LH and FSH from the anterior pituitary and subsequent activation of the gonads. The increase in levels of circulating gonadal sex steroid hormones results in development of secondary sex characteristics. Furthermore, the sex-specific brain morphology and neurochemical circuits organized during early postnatal development become activated to coordinate reproductive capacity with sexual behaviors.

Recent studies have provided much insight into the central mechanisms governing pubertal initiation in mammals. The use of laboratory animal models as sentinels for the potential impact of EDCs on human puberty should be considered with the caveat that inhibitory control over GnRH secretion during the quiescent phase differs in some ways between rodents and primates. While steroid inhibitory control represses GnRH

neurosecretory activity in rodents, this is much less potent in primates. Central mechanisms that operate independently of gonadal hormones operate to suppress GnRH in primates, as well as in rodents. Because the bulk of research on endocrine disruption is performed in rodents, most of this discussion will focus on those species. In rodents, the onset of puberty is traditionally reflected by the external markers of vaginal opening (an estrogen-mediated event) in females, and preputial separation (an androgen-mediated event) in males. In addition, in female rodents the final endpoint for pubertal onset is the age at first estrus, as it follows the first preovulatory gonadotropin surge.

A large body of evidence supports the idea that developmental exposure to environmental EDCs influences the onset and progression of puberty. Indeed, precocious (early) puberty, delayed puberty, or no change in timing of puberty, can occur in response to developmental ED exposure, depending upon the nature of the endocrine disruptor, the dose, the sex, and the timing of exposure. These studies on laboratory rodents are likely to reflect processes in humans, as epidemiological and clinical studies have shown a correlation between environmental chemical exposures and pubertal timing (Buck Louis *et al*., 2008; Wolff *et al*., 2008; Chou *et al*., 2009).

Developmental exposure to PCBs has been shown to affect the timing of puberty, as well as serum gonadotropins and hormones, with effects varying by compound and sex (summarized in **Table 3**). In contrast to the sex difference in susceptibility to other classes of EDCs, both males and females seem to be vulnerable to pubertal disruption caused by PCBs.

As mentioned earlier, PCBs are categorized into three classes based upon

arrangement of chlorine atoms around the biphenyl core. The available literature suggests that their three-dimensional structure greatly impacts their potential for disruption of pubertal onset, which is presumably related to their ability to impact interactions of hormones with their cognate steroid hormone receptors. In addition, the effects of individual PCBs may be sex-specific. Studies with the dioxin-like coplanar congener PCB126 have consistently indicated a delay in female puberty following gestational exposure over a range of doses in rats (Faqi *et al*., 1998; Muto *et al*., 2003; Shirota *et al*., 2006), while male pubertal onset is unaffected. The effects of PCB126 may be species-specific though, as pubertal onset in female goats was unaffected by gestational and lactational exposure (Lyche *et al*., 2004). Although the onset of male puberty is not affected by PCB126, a demasculinization of the AGD was observed in male rats (Faqi *et al*., 1998), and an increase in the prepubertal testosterone peak in male goats was reported (Oskam *et al*., 2005).

The effects of PCB mixtures on puberty have also been reported, including for the once commercially available Aroclors, which are named according to the average percentage chlorine of the congeners that comprise the mixture. The effects of mixtures also seem to be sex-dependent. For instance, the PCB mixture A1254 (54% chlorine content) delays the onset of puberty in gestationally- but not early postnatally-exposed male rats, while in female rats a delay in pubertal onset was noted regardless of whether exposure is restricted to gestation (Lilienthal *et al*., 2006) or early postnatal development (Sager and Girard, 1994). Delayed female puberty in response to A1254 exposure is consistently observed, even at high doses (Lee *et al*., 2007). Conversely, the more lightly

chlorinated commercial PCB mixture, A1221 (21% chlorine content), has been shown to advanced onset of puberty in female rats whether exposure occurs throughout gestation and lactation, or acutely exposed after birth (Gellert, 1978). One potential explanation for the opposite effects of A1221 and A1254 on pubertal timing in females is the difference in percentage chlorine composition of each technical Aroclor mixture. For instance, because A1254 has a greater proportion of more highly chlorinated biphenyls than A1221, these structural disparities may manifest as differences in binding affinity to hormone and neurotransmitter receptors, and their ability to act as an estrogen agonist or antagonist. While studies investigating the effects of Aroclors on puberty are informative, the effects of environmentally relevant mixtures of individual PCB congeners have also been studied. For example, our laboratory and others have observed a sex-dependent effect of puberty in rats exposed throughout the period of gestation and lactation to a reconstituted mixture of PCBs comprised of the most prevalent congeners measured in human samples. An advancement of vaginal opening and first estrus was seen in females, while a delay of preputial separation was observed in males (Colciago *et al*., 2009).

In addition to their effects on pubertal timing, PCBs have also been reported to affect serum gonadotropin and hormone levels during the peri-pubertal transition. For example, in goats gestationally exposed to PCB153, a delay in pubertal onset, reduced prepubertal serum LH and reduced serum progesterone during ovulation was observed in females (Lyche *et al*., 2004), while a decrease in serum LH was noted in males. Similar results were obtained with male goats by a separate lab. Oskam *et al*. (2005) found that PCB126 and PCB153 exposure during embryonic development caused a reduction in

prepubertal LH, an effect that persisted into the post-pubertal period (Oskam *et al*., 2005). Furthermore, that group also observed an increase in the prepubertal testosterone peak followed by a lasting reduction in testosterone for the five weeks following puberty. Effects of PCBs on serum hormones are not limited to goats; both serum estradiol and progesterone were decreased at late puberty/early adulthood (P50) in female rats gestationally exposed to PCB126 (Muto *et al*., 2003).

The question of whether early exposure to PCBs causes disruptions in human puberty remains largely unanswered (Den Hond and Schoeters, 2006). For example, epidemiologic studies have reported no association between maternal exposure to PCBs and breast development (Blanck *et al*., 2000; Gladen *et al*., 2000; Den Hond *et al*., 2002) or age at menarche in developing girls (Vasiliu *et al*., 2004; Wolff *et al*., 2008).

Table 3: PCB Effects on Peri-pubertal Neuroendocrine Endpoints

Effect	*Sex*	*Compound*	*Age at Treatment*	*Method of Exposure (Dose)*	*Age at Testing*	*Organism*	*Reference*
Neuroendocrine Gene/Protein Expression							
↓ ERβ protein in AVPV	F	A1221	E16, P1, P4	IP inj (0.34 mg/kg)	P42	Rat	(Salama *et al*., 2003)
Neuroendocrine Reproductive Function & Behavior							
↓ Sexual Receptivity and Lordosis	F	A1254	P1-P7	IP inj (2.5, 5 mg)	P60	Rat	(Chung and Clemens, 1999)
Impaired paced mating behaviors	F	A1221	E16, E18	IP Inj (0.1, 1, 10 mg/kg)	P50	Rat	(Steinberg *et al*., 2007)
Irregular estrus cycles	F	A1254	P1, 3, 5, 7, 9	Maternal gavage (32, 64 ppm)	Puberty	Rat	(Sager and Girard, 1994)
↓ Lordosis ↓ Lordosis, ↓ Sexual receptivity	F	PCB47 PCB77	E7-E18	IP inj (20 mg/kg bw; 0.25 mg, 1 mg/kg bw)	P71	Rat	(Wang *et al*., 2002)
Pubertal Onset							
Delayed pubertal onset	F	PCB126	E0-P20	Maternal gavage (3 μg/kg/day)	Puberty	Rat	(Shirota *et al*., 2006)
Delayed pubertal onset ↓ Anogenital distance	F M	PCB126	E15	Maternal gavage (10μg/kg)	Puberty	Rat	(Faqi *et al*., 1998)
Delayed pubertal onset; delayed start of regular estrous cycle; ↓ Serum estradiol and ↓ progesterone	F	PCB126	E13-E19	Maternal gavage (250 ng and 7.5 μg/kg bw)	Puberty P50	Rat	(Muto *et al*., 2003)
↑ Serum testosterone-prepubertal peak; ↓ Serum LH (PCB153 only)	M	PCB126 PCB153	E60-E150	Maternal gavage (49 ng/kg bw; 98 ng/kg bw)	12 - 40 weeks	Goat	(Oskam *et al*., 2005)
↓ Serum testosterone and LH	M	PCB126 PCB169	E7-E21	Maternal gavage (3, 30 μg/kg/day)	P42	Rat	(Yamamoto *et al*., 2005a)
↓ Prepubertal serum LH; early pubertal onset; ↑ Serum progesterone during ovulation	F	PCB153	E60-P91	Maternal gavage (98μg/kg bw)	9 months	Goat	(Lyche *et al*., 2004)
Advanced puberty Delayed	F M	PCB126, 138, 153, 180	E15-P21	Maternal Sc inj (110 mg/kg total)	Puberty	Rat	(Colciago *et al*., 2009)

testicular descent							
↓ 5alpha reductase mRNA-hypothalamus	F	PCB126, 138, 153, 180	E15-P21	Maternal Sc inj (110 mg/kg total)	P21	Rat	(Colciago *et al*., 2009)
Delayed pubertal onset	M/F	A1254	E10-E18	SC inj (30 mg/kg bw)	Puberty	Rat	(Lilienthal *et al*., 2006)
Delayed pubertal onset	F	A1254	E8 – P21	Maternal gavage (10 – 50 mg/kg daily)	Puberty	Rat	(Lee *et al*., 2007)
Delayed pubertal onset Delayed first estrus	F	A1254	P1, 3, 5, 7, 9	Maternal gavage (32, 64 ppm)	Puberty	Rat	(Sager and Girard, 1994)
↓ Tryptophan hydroxylase activity-hypothalamus	M	A1254	~P35	Gavage (0.33 mg/g bw)	~P42	Rat	(Khan and Thomas, 2004)
Slightly earlier time to pubertal onset	F	A1221	E16, P1, P4	IP inj (0.34 mg/kg)	P42	Rat	(Salama *et al*., 2003)
Early pubertal onset	F	A1221	P2, P3	SC inj (1mg/kg bw)	Puberty	Rat	(Gellert, 1978)

Abbreviations:

↑: increased; ↓: decreased; A1221: Aroclor 1221; A1254: Aroclor 1254; AGD: anogenital distance; AVPV: anteroventral periventricular nucleus of the hypothalamus; bw: Body weight; E: embryonic day; ERβ: estrogen receptor beta; F: female; FSH: follicle-stimulating hormone; GnRH: gonadotropin hormone releasing hormone; Inj: injection; IP: Intraperitoneal; M: male; mRNA: messenger RNA; No Δ: unchanged; P: postnatal day; P4: progesterone; PCB: polychlorinated biphenyl; ppm: parts per million; PR: progesterone receptor; RM: reconstituted mixture; SC: subcutaneous

SUMMARY

Taken together, the data summarized in this chapter support a role for these five classes of EDCs on development of the reproductive neuroendocrine axis. The effects of EDCs on the developing HPG axis range from disruptions of sexual differentiation of dimorphic brain regions, to alterations in gene and protein expression, enzymatic activity, serum gonadotropin and hormone levels, to interference with feedback responses between the different levels of the HPG axis. While there is limited human data, and there are inconsistencies in the available animal data, it can be concluded that the developing reproductive neuroendocrine system constitutes a sensitive target of developmental ED exposure. There appears to be increased interest in research on neuroendocrine endpoints, and we expect that the future will bring novel information, and potentially insights into mechanisms by which environmental EDCs cause neuroendocrine dysfunctions.

CHAPTER 2: EXPERIMENTAL OVERVIEW

AIMS OF THIS RESEARCH

As described in **Chapter 1**, PCBs are persistent environmental toxicants known to cause neurological and reproductive deficits in mammals (Morse *et al*., 1996; Schantz *et al*., 1996; Tilson and Kodavanti, 1997; Hany *et al*., 1999; Guo *et al*., 2000; Gore, 2001a). The developing offspring of exposed pregnant females are particularly vulnerable to endocrine-disrupting effects of PCBs and other toxicants, because the critical period of brain sexual differentiation occurs in late fetal/early postnatal life, and disruption of normal development during this period can be devastating to adult sexual functions. The overarching goal of this dissertation research project is to characterize the mechanisms through which perinatal PCB exposure disrupts sexual differentiation of the hypothalamus. My focus is a sexually dimorphic nucleus whose morphology, neurochemistry, and expression of nuclear hormone receptors correlate with the control of adult reproductive physiology and behavior: the anteroventral periventricular nucleus (AVPV). Sex differences in this region occur due in part to developmental apoptosis, and are organized by perinatal gonadal steroid hormones. Embryonic levels of sex steroid hormones contribute not only to the observed sex differences in volume and cell number in this region during adulthood (Rhees *et al*., 1990b; Sumida *et al*., 1993), but also to the development of appropriate sex-specific neuroendocrine physiology and behavior. **The specific hypothesis behind the research is that perinatal PCB exposure disrupts sexual differentiation of the developing AVPV via alterations in developmental**

apoptosis, gene and protein expression, ultimately resulting in reproductive dysfunction during adulthood. This hypothesis is based on the following observations: 1) prenatal PCB exposure causes endocrine disrupting effects at the hypothalamic level of the reproductive axis in vertebrate species, including mammals (Khan *et al*., 2001; Lichtensteiger, 2003; Salama *et al*., 2003; Colciago *et al*., 2006); 2) PCBs induce apoptosis in neuronal cultures (Hwang *et al*., 2001; Mariussen *et al*., 2002; Howard *et al*., 2003; Sanchez-Alonso *et al*., 2003; Sanchez-Alonso *et al*., 2004); 3) perinatal exposures to substances that act on steroid hormone receptors change apoptosis in a sex-specific manner (Ito *et al*., 1986; Murakami and Arai, 1989; Nishizuka *et al*., 1993; Sumida *et al*., 1993; Arai *et al*., 1994), although this latter question has not yet been addressed specifically for PCBs. The specific aims are designed to provide a comprehensive assessment of the PCB-induced alterations in normal development of the sexually dimorphic neuroendocrine system.

Specific Aim I: Test the hypothesis that environmentally persistent PCB congeners have neurotoxic effects on the hypothalamic GT1-7 cell line *in vitro*. Selection of congeners and doses for *in vivo* experiments in Aims II and III are informed by these data.

a) Evaluate whether treatment with representative PCBs affect GnRH peptide levels in GT1-7 cells.
b) Assess whether PCBs induce cell death in the GT1-7 cell line as indicated by loss of cell viability and abnormal cell and nuclear morphology. If cell death is induced, establish whether death is due to apoptosis or necrotic cell death.

Specific Aim II: Test the hypothesis that prenatal exposure to PCBs has sexually dimorphic effects on the developing hypothalamus of male and female Sprague-Dawley rats, and ascertain the mechanisms for these effects.

a) Determine whether prenatal exposure to PCBs changes developmental apoptosis in a sexually dimorphic manner in the AVPV, and, if so, determine whether those changes manifest as altered adult volume of the AVPV at P60.
b) Investigate whether PCB exposure alters gene expression of GnRH and nuclear hormone receptors in the preoptic area at P1 and P60.
c) Establish whether PCBs affect protein expression of the nuclear hormone receptor ERα in the developing and adult hypothalamus. To this end, the numbers of cells in the AVPV and MPN that express estrogen receptor alpha (ERα) will be counted in males and females at P1 and P60.

Specific Aim III: Test the hypothesis that prenatal PCB exposure has long-term physiological consequences on reproductive development and physiology.

a) Determine whether pubertal endpoints in rats (body weight, anogenital distance, age at vaginal opening and onset of estrous cycles for females, and preputial separation for males) are significantly different between PCB- and vehicle-treated animals.
b) Determine whether PCB exposure alters normal estrous cyclicity in females.

c) Determine whether serum hormone levels (estradiol, testosterone, progesterone, luteinizing hormone) are altered during adulthood.

GENERAL RESEARCH METHODS

There are three major experiments in my dissertation. Detailed methods for each experiment are described in each chapter.

Aim I:

PCB congeners are classified into three categories, coplanar, dioxin-like coplanar, and non-coplanar, based upon arrangement of chlorine substituents around the biphenyl core. Congeners with no more than one chlorine atom in an *ortho* position may assume a coplanar configuration. Congeners that have no more than one chlorine atom in the *ortho* positions, a total of four or more chlorine substituents, both *para* positions (4 and and 4') chlorinated, and two or more chlorinated *meta* positions (3, 3', 5, and 5'), are classified as dioxin-like coplanar. Because these congeners bind to the aryl hydrocarbon (Ah) receptor, they are associated with toxicities similar to dioxins (Safe, 1994). More than one chlorine substituent in the ortho position results in steric hindrance of ring rotation; these congeners are classified as noncoplanar. While all three classes persist in the environment, the non-coplanar class is found in the highest concentrations in both human and wildlife samples (Gladen *et al*., 2003). As our model, we use the hypothalamic GT1-7 cells, which synthesize and secrete GnRH, to establish the mechanisms of neurotoxicity exerted by individual PCBs of the three classes. GT1-7 cells (generously provided by Dr.

Pamela Mellon, UCSD (Mellon, 1990) will be treated with PCB74 (coplanar), PCB118 (dioxin-like coplanar), or PCB138 (noncoplanar), in a dose-response and time-course design. Cells are assessed for GnRH peptide, morphology, viability, and DNA fragmentation. See detailed methods for *in vitro* experiments in Chapter 3.

Figure 2.1: Experimental overview of *in vitro* studies

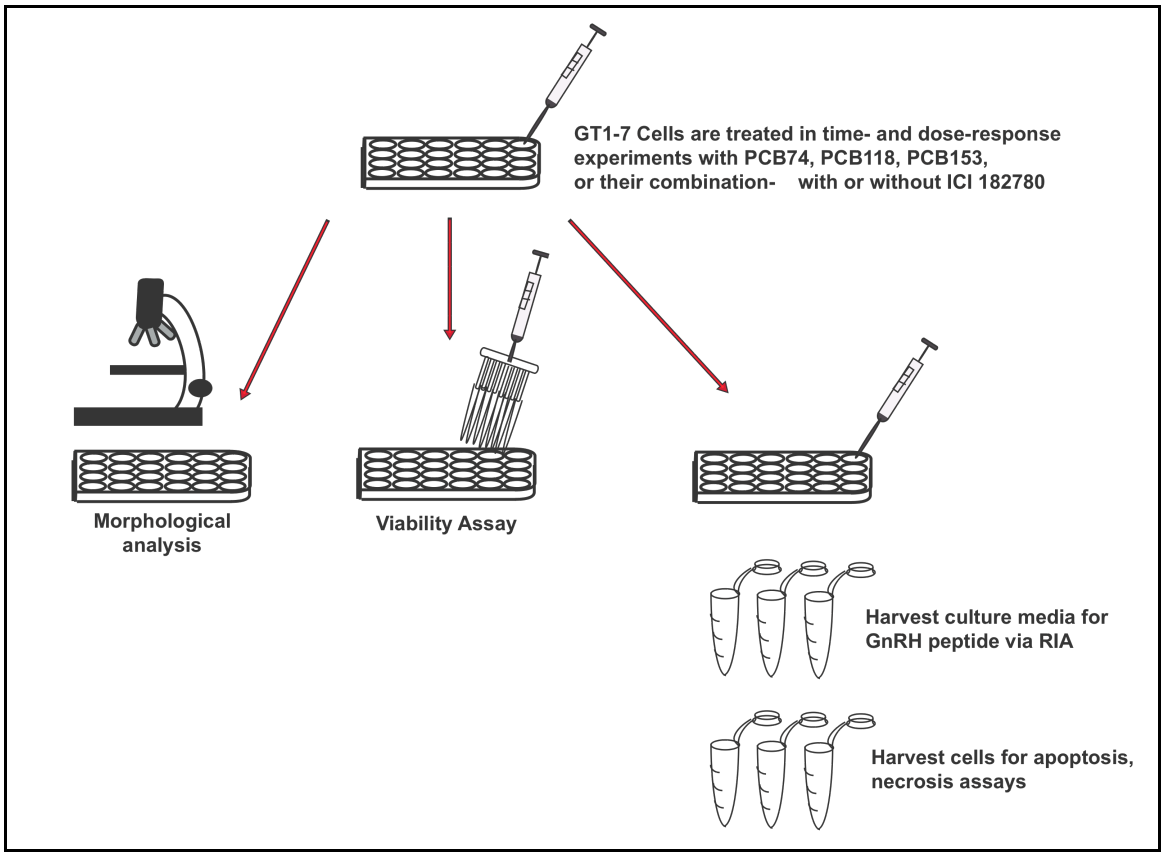


Figure 2.1: GT1-7 cells grown in culture are treated in time- and dose-response experiments with representative PCBs from each classification (coplanar, dioxin-like coplanar, non-coplanar) alone or in combination, with or without estrogen receptor antagonist. At each timepoint, cells are either qualitatively evaluated for morphology, or harvested for viability, apoptosis, or necrosis assays. Cell culture media is harvested at each timepoint to assay GnRH peptide.

Aim II:

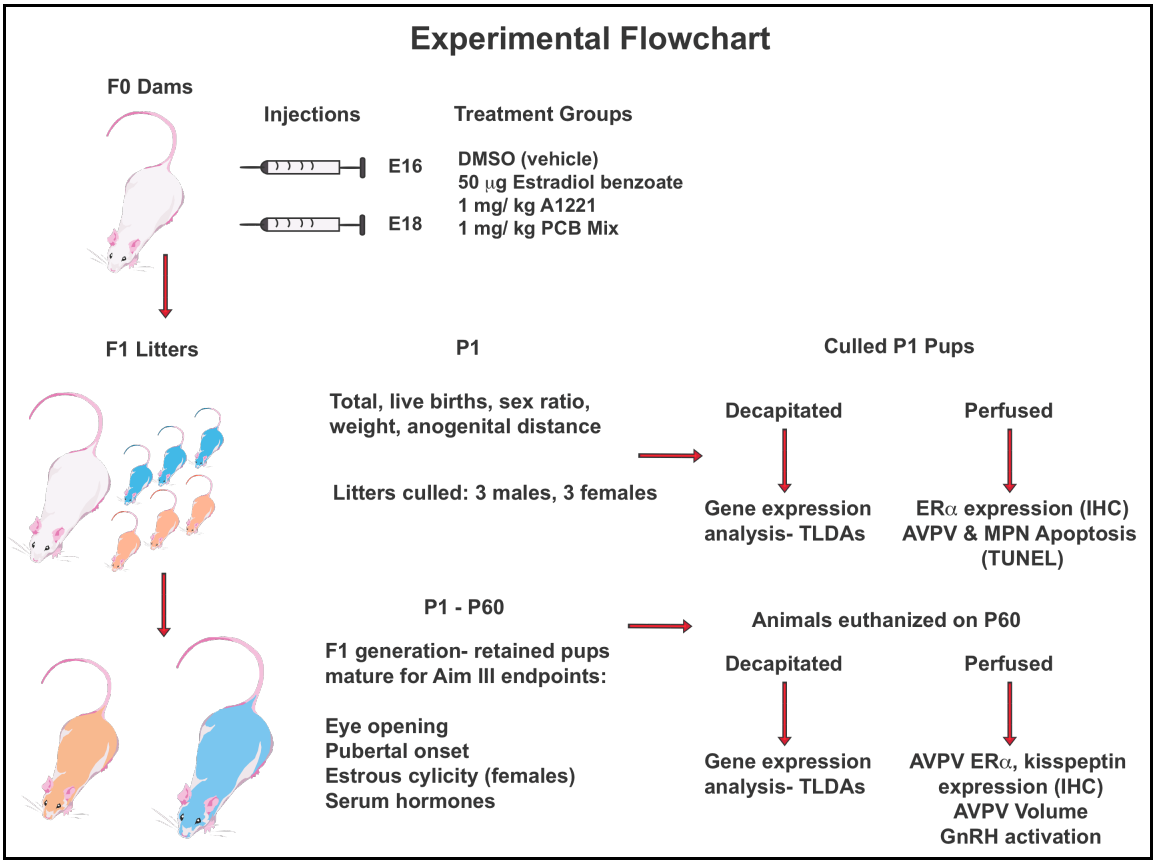
Gender differences in gonadal steroid hormones during perinatal development influence developmental neuronal apoptosis, and contribute to the morphological differences between male and female AVPV. The volume, cell number, and functional neurochemistry of this brain region are particularly sensitive to small changes in hormone status. Perinatal exposures to substances that act on steroid hormone receptors change apoptosis in a sex-specific manner (Ito *et al*., 1986; Murakami and Arai, 1989; Nishizuka *et al*., 1993; Sumida *et al*., 1993; Arai *et al*., 1994), although this has not yet been addressed specifically for PCBs. Because PCBs can exert anti-estrogenic activity via competitive binding of the estrogen receptor (Bonefeld-Jorgensen *et al*., 2001; Salama *et al*., 2003), and are known to alter sex steroid hormone levels in gestationally exposed neonatal rodents (Kaya *et al*., 2002; Yamamoto *et al*., 2005a), they may interfere with processes that are regulated by sex hormones, such as sexual differentiation of the brain.

The choice of age for investigation of apoptosis, P1, was based on previous evidence showing sex differences in the extent of apoptosis in the AVPV at this age (Sumida *et al*., 1993; Davis *et al*., 1996a; Yoshida *et al*., 2000). P60 was selected as an age for investigation of the impact of altered developmental apoptosis on the adult volume of the AVPV because by this age, the sex differences in the size, cell number and expression of nuclear hormone receptors are established. The selection of the POA for investigation of gene expression is based on the location of the AVPV within the POA, and the relative ease of performing the POA microdissection compared to the technical

difficulty of isolating the AVPV. Thus, whether prenatal exposure to PCBs alters the extent of developmental apoptosis, gene expression, and expression of ERα in a gender-dependent manner in the AVPV of the postnatal rat hypothalamus, leading to permanent alterations in the adult brain will be assessed.

Timed-pregnant Sprague-Dawley rats will be injected intraperitoneally on E16 and E18 with either vehicle (sesame oil), 50 μg estradiol benzoate, or 1 mg/kg A1221, or 1 mg/kg of a reconstituted mixture of PCB138, PCB153, and PCB180. This treatment timing falls after the differentiation of cells in the AVPV and MPN, and before developmental apoptosis begins. Based on the maternal dose, each pup will be exposed to an estimated 2 μg/kg PCB, which falls within the range of human neonatal exposures (Lackmann, 2002). For each experiment, gonadally-intact male and female rats gestationally and lactationally exposed to PCBs will be used at P1 and P60. Animals from Aim II will be allowed to mature to puberty for Aim III, and will be euthanized at P60 (**Figure 2.2**).

Figure 2.2: Experimental flowchart for *in vivo* studies.



Aim III:

In addition to cellular and molecular effects, PCBs may cause permanent changes that are manifested during the pubertal process, during which endogenous steroid hormone production increases and activates the sex-specific brain morphology and neurochemistry organized during early postnatal life. Thus, precocious (early) puberty, delayed puberty, or no change in timing of puberty, can occur in response to developmental PCB exposure. Preputial separation (PPS) in males and vaginal opening (VO) in females are reflections of the increase in gonadal steroid hormone secretion associated with puberty. These endpoints have been historically used as a general indicator of the progression from adolescence to adult reproductive capacity (Korenbrot *et al*., 1977; Safranski *et al*., 1993), although neither is a definitive marker that adult reproductive capacity has been attained. Reproductive maturity occurs shortly after VO and PPS in rats, and these endpoints are useful markers that rats are within the peripubertal stage of development. In addition, the anogenital distance (AGD) is a useful marker of masculinization/defeminization (Marois, 1968), and can be an indicator of endocrine disruption. Therefore, the goal of Aim III is to ascertain the repercussions of prenatal PCB treatment on the attainment and manifestation of adult reproductive functions. Thus, whether pubertal endpoints in rats (body weight, anogenital distance, age at vaginal opening and onset of estrous cycles for females, preputial separation for males, and serum hormones) are significantly different between PCB- and vehicle-treated animals is assessed.

SUMMARY:

Exposure to EDCs during the vulnerable developmental period can result in profound neurological and reproductive deficits. As PCBs are pleiotropic compounds that exert estrogenic or anti-estrogenic activity via competitive binding of the estrogen receptor, they may interfere with processes that are regulated by sex hormones, such as sexual differentiation of the brain. The studies outlined above are designed to provide new insight into the mechanisms responsible for the neuroendocrine toxicity of PCBs, and may form the basis for therapeutic strategies to prevent or ameliorate reproductive deficits resulting from perinatal PCB exposure.

CHAPTER 3: CELL DEATH MECHANISMS IN GT1-7 GNRH CELLS EXPOSED TO POLYCHLORINATED BIPHENYLS PCB74, PCB118, AND PCB153

The text in this section is excerpted from the article "Cell death mechanisms in GT1-7 GnRH cells exposed to polychlorinated biphenyls PCB74, PCB118, and PCB153," Dickerson SM, Cunningham SL, and Gore AC, in *Toxicology & Applied Pharmacology* (2009), with permission from the journal.

ABSTRACT

Exposure to endocrine-disrupting chemicals (EDCs) such as polychlorinated biphenyls (PCBs) causes functional deficits in neuroendocrine systems. We used an immortalized hypothalamic GT1-7 cell line, which synthesizes the neuroendocrine peptide gonadotropin-releasing hormone (GnRH), to examine the neurotoxic and endocrine disrupting effects of PCBs and their mechanisms of action. Cells were treated for 1, 4, 8, or 24 h with a range of doses of a representative PCB from each of three classes: coplanar (2,4,4',5-tetrachlorobiphenyl: PCB74), dioxin-like coplanar (2',3,4,4',5' pentachlorobiphenyl: PCB118), non-coplanar (2,2',4,4',5,5'-hexachlorobiphenyl: PCB153), or their combination. GnRH peptide concentrations, cell viability, apoptotic and necrotic cell death, and caspase activation were quantified. In general, GnRH peptide levels were suppressed by high doses and longer durations of PCBs, and elevated at low doses and shorter time points. The suppression of GnRH peptide levels was partially

reversed in cultures co-treated with the estrogen receptor antagonist ICI 182,780. All PCBs reduced viability and increased both apoptotic and necrotic cell death. Although the effects for the three classes of PCBs were often similar, subtle differences in responses, together with evidence that the combination of PCBs acted slightly differently from individual PCBs, suggest that the three tested PCB compounds may act via slightly different or more than one mechanism. These results provide evidence that PCB congeners have endocrine disrupting and/or neurotoxic effects on the hypothalamic GnRH cell line, a finding that has implications for environmental endocrine disruption in animals.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a family of synthetic organic pollutants that contaminate both urban and rural environments. These compounds were widely used for industrial applications between the 1930s and 1970s, due to their stability, non-flammability and high dielectric constant. The same properties that made PCBs desirable for industrial use contribute to their resistance to degradation and to their toxicity. Despite the banning of PCB manufacture in the United States in 1977, humans are still exposed via food, and have appreciable body burdens (Stellman *et al*., 1998).

PCBs can act as endocrine disrupting chemicals (EDCs) via their interactions with sex steroid hormone receptors, steroidogenic enzymes, and other mechanisms [reviewed in (Dickerson and Gore, 2007)]. Indeed, PCBs have been shown to perturb endocrine and

reproductive systems in a variety of species (Khan and Thomas, 2001; Salama *et al*., 2003; Khan and Thomas, 2004). Further, PCBs have been proposed to disrupt neuroendocrine cells in the hypothalamus, including cells that synthesize and release the neurohormone, gonadotropin-releasing hormone (GnRH) [reviewed in (Dickerson and Gore, 2007; Gore, 2008a)]. The decapeptide GnRH is released from neuroterminals in the hypothalamus into the portal capillary system leading to the anterior pituitary gland, where GnRH acts to stimulate synthesis and release of gonadotropins into the general circulation. These two hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) act at the gonads to stimulate gonadal maturation, steroidogenesis and gametogenesis. Although all three levels of the hypothalamic-pituitary-gonadal axis must function properly in order for reproductive function to occur, the hypothalamic GnRH neurons provide the driving force upon this system.

PCBs have other actions on the nervous system beside neuroendocrine GnRH cells. They target neurotransmitter receptors and biosynthetic enzymes (Seegal *et al*., 1991b; Seegal *et al*., 1991a; Juarez de Ku *et al*., 1994; Altmann *et al*., 2001; Mariussen *et al*., 2002; Gafni *et al*., 2004; Coccini *et al*., 2006), and they act upon steroid hormone receptors in the brain (Dickerson and Gore, 2007). Importantly, GnRH neuroendocrine cells are regulated by a complex interplay of neurotransmitters and steroid hormones. Thus, PCBs may exert both direct and indirect actions upon GnRH neurons, thereby disrupting hypothalamic-pituitary-gonadal function.

The mechanisms of action and the degree of toxicity of PCBs are dependent upon degree of chlorination, three-dimensional structure, timing and duration of exposure,

dosage, and cell type (Kodavanti, 2005; Ruiz *et al*., 2008). PCBs are grouped into three general categories: coplanar, dioxin-like coplanar, and non-coplanar, with differential effects on cell death, including apoptosis and necrosis as shown in neuronal hippocampal or cortical cell cultures or immortalized neuronal cell lines (Hwang *et al*., 2001; Inglefield *et al*., 2001; Kang *et al*., 2001; Howard *et al*., 2003; Sanchez-Alonso *et al*., 2003; Lee *et al*., 2004; Sanchez-Alonso *et al*., 2004; Costa *et al*., 2007). However, the neurotoxicity of PCBs has not been addressed for neuroendocrine cells. Although previous work (Gore *et al*., 2002) has shown that PCBs can alter release of GnRH and change GnRH gene expression in the GT1-7 immortalized hypothalamic cell line (Mellon *et al*., 1990), the mechanisms of neurotoxicity have never been studied or quantified. Here, we hypothesized that the three different classes of PCBs (coplanar, dioxin-like coplanar, non-coplanar) would cause a differential endocrine disruption of GnRH release, and induce differing levels of apoptosis or necrosis in GT1-7 hypothalamic cells. We also evaluated effects of a mixture of the three PCBs in order to assess whether there were additive or synergistic effects on these endpoints. Finally, we examined the role of the nuclear estrogen receptor in mediating effects of PCBs on GnRH release by co-administering PCBs with an estrogen receptor antagonist, ICI 182,780.

METHODS

Chemicals

We purchased 2,4,4',5-tetrachlorobiphenyl (CAS RN: 32690-93-0; PCB74- 99.9% purity; lot no. 102293), 2',3,4,4',5' pentachlorobiphenyl (CAS RN: 31508-00-6; PCB118- 99.9% purity; lot no. 961002LB-AC), and 2,2',4,4',5,5'-Hexachlorobiphenyl (CAS RN: 35065-27-1; PCB153- 99.9% purity; lot no. 062703JR-AC), from Accustandard (New Haven, CT, USA). Dimethylsulfoxide (DMSO), and staurosporine (positive control for apoptotic cell death) were purchased from Sigma (St. Louis, MO, USA) and 4',6-diamidino-2-phenylindole (DAPI) was purchased from Calbiochem (La Jolla, CA, USA). ICI 182,780 was purchased from Tocris Bioscience (Ellisville, MO, USA). We purchased phenol red-free Dulbecco's Modification of Eagle's Medium (DMEM), fetal calf serum, L-glutamine, penicillin, and streptomycin from CellGro (MediaTech, Inc., Herndon, VA, USA). The CellTiter-Blue™ Cell Viability and APO-One Homogeneous Caspase-3/7 Activation assay was purchased from Promega Corporation (Madison, WI, USA). Anti-mouse cleaved caspase-3 and anti-mouse cleaved caspase-9 antibodies were from Cell Signaling Technology, Inc. (catalog no. 9664 and 9509, respectively; Beverly, MA, USA), while anti-mouse cleaved caspase-8 antibody was obtained from R&D Systems, Inc. (catalog no. AF1650; Minneapolis, MN, USA).

GT1-7 cell culture and maintenance

GT1-7 cells (kindly provided by Dr. Pamela Mellon; (Mellon *et al*., 1990) were maintained in phenol red-free DMEM supplemented with 10% heat-inactivated fetal calf

serum (FCS) and antibiotics (100 U/ml, penicillin, 100 mg/ml streptomycin). Cells were grown at 37°C with 5% CO_2 as described previously (Gore, 2002; Gore *et al*., 2002), and used between passages 15 – 20. Cells were sub-cultured into six-well or 96-well tissue culture dishes 2–3 days before experiments in Dulbecco's minimum essential medium (DMEM) plus FCS and antibiotics, and grown to approximately 60–70% confluency. To avoid growth hormone or steroid effects of FCS, we switched to serum-free medium four hours before experiments. All experiments were performed on triplicate monolayers of either approximately $1X10^6$ cells for six-well plates or 50,000 cells for 96-well plates, and were repeated three to four times on separate culture dishes.

PCB congener treatments

GT1-7 cells were treated with either vehicle (DMSO), individual PCBs [PCB74, PCB118 or PCB153 (each at 0.1, 1, 10, 100 μM)] or a PCB Mix [1:1:1 mixture of PCB74, PCB118, and PCB153 (0.1, 1, 10, 100 μM final additive molar equivalent of individual PCBs)]. For some experiments, staurosporine (200 nM; Sigma) was used as a control for apoptotic cell death (Koh *et al*., 1995). We diluted all drugs in DMSO and used them at a final dilution of 1:1000 in medium. The vehicle was applied at a 1:1000 dilution. In separate experiments, cells were co-administered 1000 nM ICI 182,780. We maintained cultures at 37°C with 5% CO_2 after application of drugs. Following 1, 4, 8, or 24 h of treatment, cells were viewed on an inverted phase contrast microscope at a magnification of X250. Photomicrographs were imaged using a Nikon digital camera mounted on the microscope.

GnRH Peptide Assay

GnRH peptide levels in the medium were measured in two double-antibody radioimmunoassays with duplicate samples of 100 μl. For each experiment, media from triplicate cultures were pooled into a single sample. Three pooled samples per experiment were analyzed, and the experiment was repeated four times. The antibody to GnRH was provided by Dr T. Nett (R1245). Synthetic GnRH used as trace and standard was purchased from Richelieu Laboratories (Montreal, Canada). Assay sensitivity was 0.1 pg/tube at 95% binding. The intra-assay coefficients of variation were 3.4% and 3.9%, and the inter-assay coefficient of variation was 3.1%.

Determination of Cell Viability

We used the CellTiter-Blue® Cell Viability Assay (Promega Corporation) to measure the viability of cells after PCB treatment. This fluorometric assay is based on the metabolic capacity of viable cells to reduce resazurin into resorufin, which is highly fluorescent at 590 nm. Nonviable cells lose metabolic capacity to reduce the indicator dye, and therefore do not generate a fluorescent signal. Briefly, the assay was carried out according to the manufacturer's recommendations (Promega). Cells were treated with PCB or vehicle for durations between 0 and 24 hours. CellTiter-Blue® Reagent was added at a volume of 20 μL to each well one hour before fluorescence was recorded. The fluorescence of the samples was measured at 590 nm on a DTX 880 Multimode Plate Reader (Beckman-Coulter, Inc., Fullerton, CA). The presence of PCBs in the culture media did not interfere with the viability assay procedures.

Detection of cell death

To detect the presence of necrotic cell death, treated cell populations were trypsinized and resuspended in a 1:1 mixture of 1X PBS and 0.4% Trypan Blue solution (Sigma) and counted under an inverse phase contrast microscope with a Neubauer improved hemacytometer (Hausser Scientific, Horsham, PA). Cells with an intact membrane (viable or apoptotic cells) are not permeable to Trypan Blue and remained unstained, while cells with a damaged membrane (necrotic cells) stain blue. Necrotic cell death is expressed as the percentage stained cells per total cell number counted. Three replicates per experiment were analyzed, and the experiment was repeated four times.

DAPI Staining of Apoptotic Nuclei

Following fixation with 4% paraformaldehyde, GT1-7 cells grown on 22 mm glass cover slips were rinsed for 5 minutes with PBS, and stained with 1 μg/ml DAPI in water for 20 min at room temperature. We then removed excess DAPI by a rinse with water, and mounted cover slips onto slides with VectaShield (Vector Laboratories, Burlingame, CA, USA). We characterized nuclear morphology and quantified chromatin condensation using an Olympus BX61 System fluorescence microscope at a magnification of 400X. Normal cells have a smooth, round nucleus characterized by faint staining. Apoptotic cells were identified by the presence of brightly labeled pyknotic nuclei. The number of neurons with condensed nuclei vs. the number of neurons with intact nuclei was scored in five randomly chosen microscopic fields (each field typically contained 25 – 50 cells). We counted a total of at least 200 cells per slide, and then

reported the number of apoptotic cells as a percentage. Three cultures were analyzed per experimental condition for each experiment, and experiments were repeated three times.

DETERMINATION OF CASPASE ACTIVATION

The Apo-ONE Homogeneous Caspase-3/7 Detection system (Promega Coropration, Madison, WI) was used to determine whether caspase-3/7 was activated following treatment with the toxicants, using the protocol provided by the manufacture. Following incubation at room temperature for three hours, caspase-3/7 activation were estimated from the fluorescence of each sample at the excitation wavelength at 485 nm and the emission wavelength at 535 nm using a fluorescence reader DTX 880 Multimode Detector (Beckman-Coulter, Inc., Fullerton, CA).

To visualize activated (cleaved) caspases -8 or -9, GT1-7 cells grown on top of 22 mm glass coverslips in 6-well tissue culture were fixed in 4% paraformaldehyde for 10 minutes at 4°C and then rinsed in PBS. Non-specific binding of endogenous proteins to primary antibody was blocked by incubating coverslips in 5% normal goat serum and 5% bovine serum albumin in PBS for 1 hour at room temperature. Cells were incubated for 1 hour at room temperature with rabbit polyclonal antibodies against mouse active caspase-8 or caspase-9. Following a rinse in PBS and incubation in an anti-rabbit secondary antibody conjugated to fluorescein (Vector Labs, Burlingame, CA, USA), slides were mounted with VectaShield mounting media (Vector Labs, Burlingame, CA, USA) and visualized on an Olympus BX61 System fluorescence microscope. Observations of the

presence or absence of activated caspase staining were made for each treatment and time point.

Statistical analysis of results

The effects of each PCB were analyzed by two-way ANOVA. Variables for most studies were dose and duration. For the ICI 182,780 experiment, variables were treatment (PCB alone or PCB plus ICI 182,780) and dose. Experiments were performed identically for each collection on the triplicate cultures. Results of triplicate cultures for each experiment were considered to be one independent variable; and thus for statistical purposes, final N's were 3 – 4 per group. When a significant effect was observed, post-hoc comparisons were performed using Fishers PLSD/Tukey-b analysis. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

GnRH peptide levels:

The GT1-7 cell media were collected 1, 4, 8 or 24 hours following treatment with PCBs 74, 118, 153, or their combination (referred to hereafter as PCB Mix). For each individual PCB and the PCB Mix, two-way ANOVA showed significant main effects of both dose ($p < 0.005$) and duration ($p < 0.005$), as well as an interaction of dose and duration ($p < 0.005$). In general, GnRH peptide levels were higher in the PCB treatment groups when compared to corresponding vehicle treatment at early timepoints (1 and 4

hours; **Figure 3.1**) and lower in PCB-treated cultures at later time points (8 and 24 hours; **Figure 3.2**).

Figure 3.1: PCB Effects on GnRH Peptide Release in GT1-7 Cells

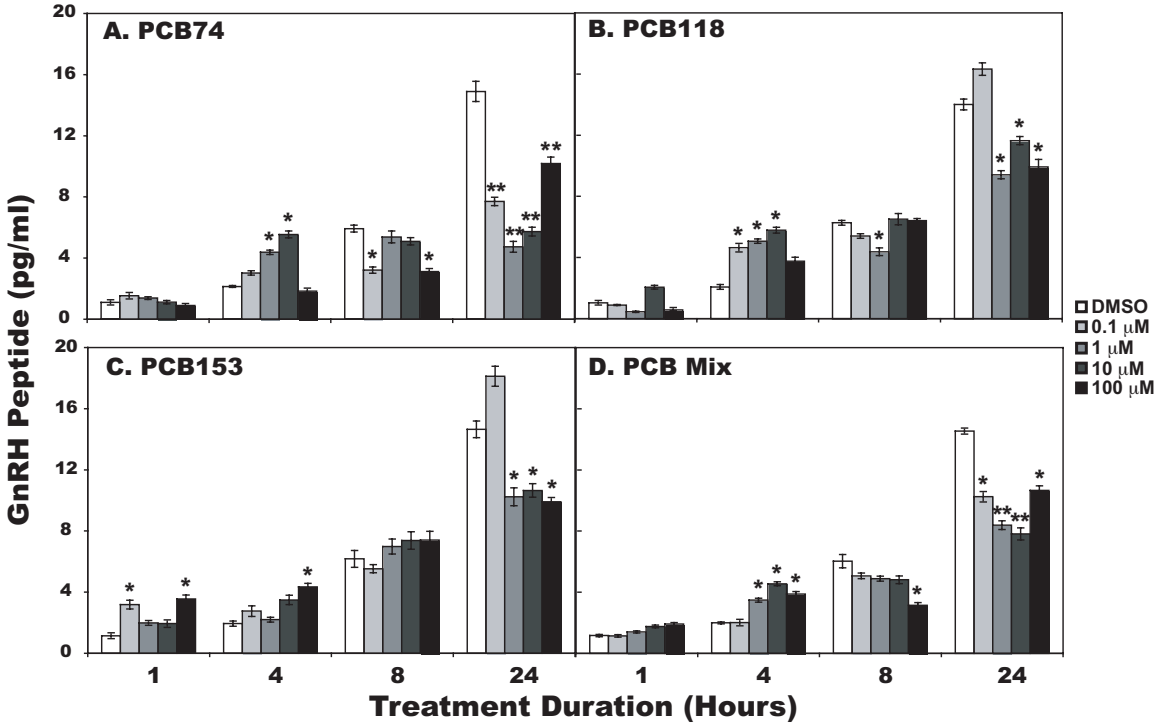


Figure 3.1. The effects of (A) PCB74, (B) PCB118, (C) PCB153, or (D) PCB Mix on GT1-7 GnRH peptide levels. The cells, seeded on 96-well plates, were treated for 1, 4, 8, and 24 hrs with 0.1% DMSO alone (vehicle) or with 0.1, 1, 10, or 100 mM PCB. By 4 hr post-treatment, a stimulation of peptide was observed in PCB-treated cells in a dose-dependent manner that varied by compound. At late time points (8 hr, 24 hr) PCBs suppressed GnRH peptide. Results are expressed as average GnRH peptide levels ± S.E.M for each experimental average of pooled triplicate cultures (N = 4) per treatment. *$p < 0.05$, **$p < 0.005$ vs. vehicle (DMSO).

The nuclear estrogen receptor antagonist ICI 182,780 (1000 nM) was co-administered with PCB74, PCB118, PCB153, or PCB Mix (1 μM dose, 24 hour timepoint) to ascertain whether endocrine-disrupting effects of PCBs are manifested upon GnRH release. Significant differences between groups were detected by ANOVA ($p < 0.001$). Post-hoc analysis demonstrated that ICI alone reduced ($p < 0.05$) GnRH peptide levels in control cultures (Figure 3.1). PCB treatment was associated with suppressed GnRH peptide levels ($p < 0.01$), which was partially reversed by co-treatment with ICI 182,780 ($p < 0.001$ compared to PCB with no ICI 182,780; **Figure 3.2**).

Figure 3.2: ER Modulates PCB-Suppression of GnRH Peptide

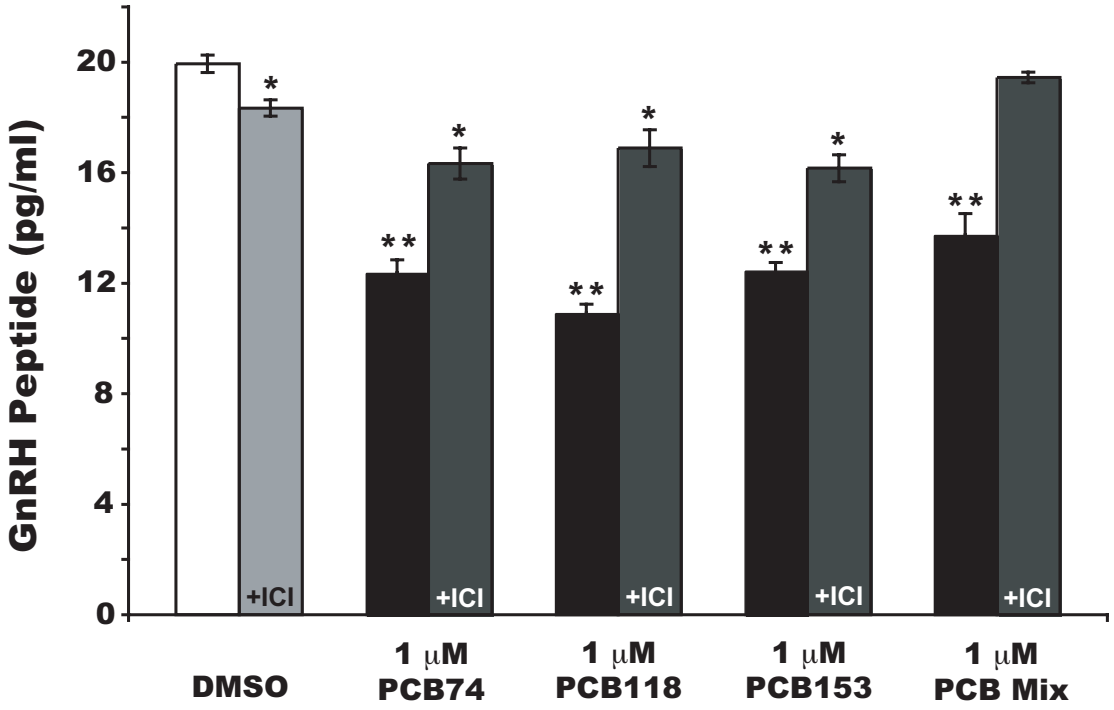


Figure 3.2. **Estrogen receptor antagonism modulates PCB-suppression of GnRH peptide release.** Cells were treated for 24 hrs with 0.1% DMSO alone (vehicle), 1000 nM ICI alone, 1 μM PCBs, or 1 μM PCBs coadministered with ICI. Coadministration of PCB with ICI partially reversed PCB-induced suppression of GnRH peptide. Results are expressed as average GnRH peptide levels ± S.E.M for each experimental average of pooled triplicate cultures (N = 4) per treatment. *$p < 0.05$, **$p < 0.005$ vs. vehicle (DMSO).

GT1-7 cell viability:

GT1-7 cell viability was measured by the CellTiter-Blue® Cell Viability Assay. We noted no significant differences in cellular viability from 0 to 24 h of exposure between untreated cultures and cultures treated with DMSO, the vehicle for PCBs (data not shown). For each PCB, two-way ANOVA showed significant main effects of both dose ($p < 0.005$) and duration ($p < 0.005$), as well as an interaction of dose and duration ($p < 0.005$). Effects varied for the individual congeners, but in general, PCB74 and PCB118 had inhibitory effects on relative cell viability at the intermediate doses and at all doses by the 24-hour timepoint (**Figure 3.3A and 3.3B).** PCB153 affected cell viability at varying doses depending upon timepoint (**Figure 3.3C**). The PCB Mix had the least effect on viability (**Figure 3.3D**).

Figure 3.3: PCB Effects on GT1-7 Cell Viability

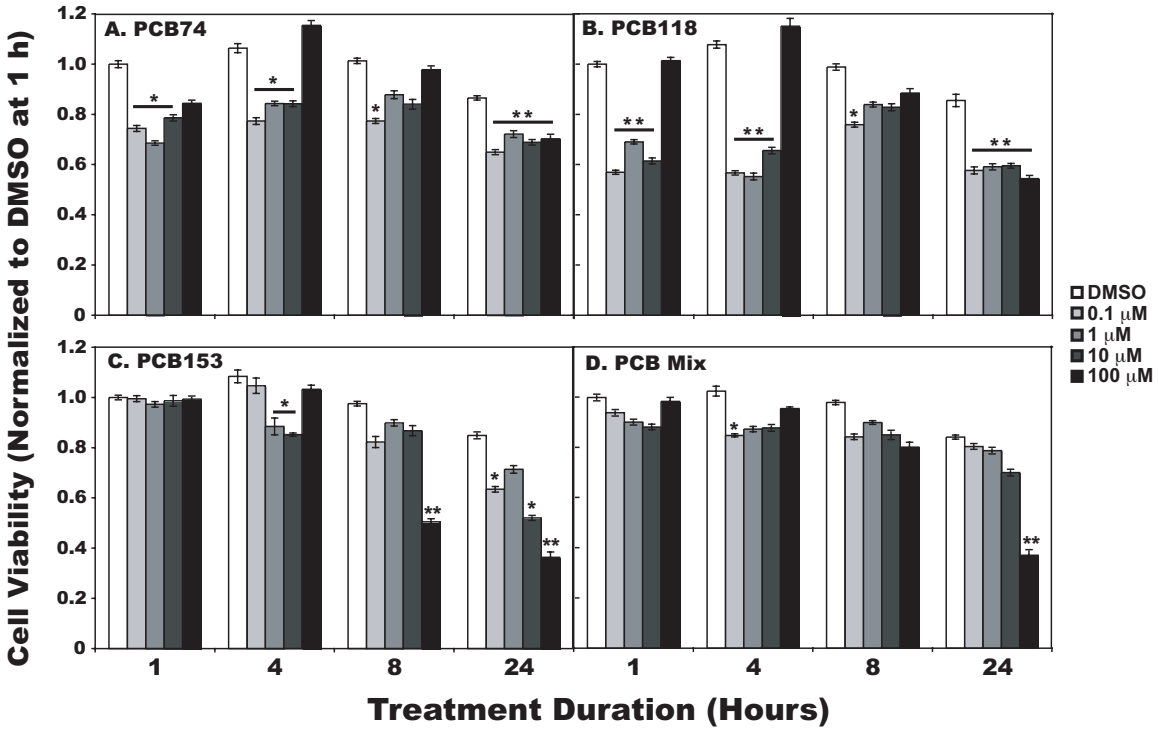


Figure 3.3. The effects of (A) PCB74, (B) PCB118, (C) PCB153, and (D) PCB Mix on GT1-7 cell viability. Fluorescence measured at $560_{Ex}/590_{Em}$ is proportional to the number of metabolically active cells. Samples at each timepoint were normalized to vehicle control fluorescence at 1 hr timepoint, and are expressed as percentage control. Individual congeners had inhibitory effects upon cell metabolism/viability vs. vehicle at 1 hr, whereas their combination only differed from control at the lowest dose at 4 hr. Each value, expressed as percentage control, is the mean ± S.E.M of four experiments run in triplicate. After 24 hrs treatment, all PCBs decreased viability. *$p < 0.05$, **$p < 0.005$ vs. vehicle (DMSO) at 1 hr baseline.

Necrotic and apoptotic cell death:

To differentiate between effects of PCBs on necrotic of apoptotic cell death, Trypan blue staining (**Figure 3.4A**) or DAPI labeling (**Figure 3.4B**) were used. Quantification of necrotic cell death (**Figure 3.5)** showed that PCBs caused little necrosis until the 8 hour timepoint, at which the highest concentration of PCB118 or PCB153 caused a significant increase. After 24 h treatment, all of the PCBs caused a significant increase in necrotic cell death.

We also examined the effects of PCBs on apoptotic cell death (**Figure 3.4B, Figure 3.6**). Staurosporine, a protein kinase inhibitor that induces apoptosis in neuronal cells (Krohn *et al*., 1998), served as a positive control. Staurosporine consistently induced cell death in GT1-7 cells (data not shown). For the PCBs, by 4 hours of exposure, all of the PCBs induced an increase in the percentage of apoptotic nuclei, and PCB118 and the PCB Mix had effects at the 1-hour timepoint. Notably, the low and intermediate concentrations of PCBs were most likely to induce apoptosis for all of the PCBs, with the highest concentrations having the least effect (**Figure 3.6**).

Caspase activation was assessed at the 4-hour timepoint. For the caspase-3/7 assay, PCB74, PCB118, and the PCB Mix had similar effects, with the low and intermediate concentrations of PCBs causing about a 2-fold increase in caspase-3/7 activity **(Figure 3.7).** PCB153 did not significantly affect caspase-3/7 activity (**Figure 3.7).**

Immunofluorescence for cleaved (activated) caspases -8, and -9 were visualized by fluorescence microscopy. Treatment with PCBs resulted in detection of activated caspase-9 in all PCB treatments but not the vehicle (**Figure 3.4C, 3.4D**). No immunolabeling was observed for caspase-8 for any of the treatments (**Figure 3.4E, 3.4F**).

Figure 3.4: PCBs Activate Markers of Apoptosis

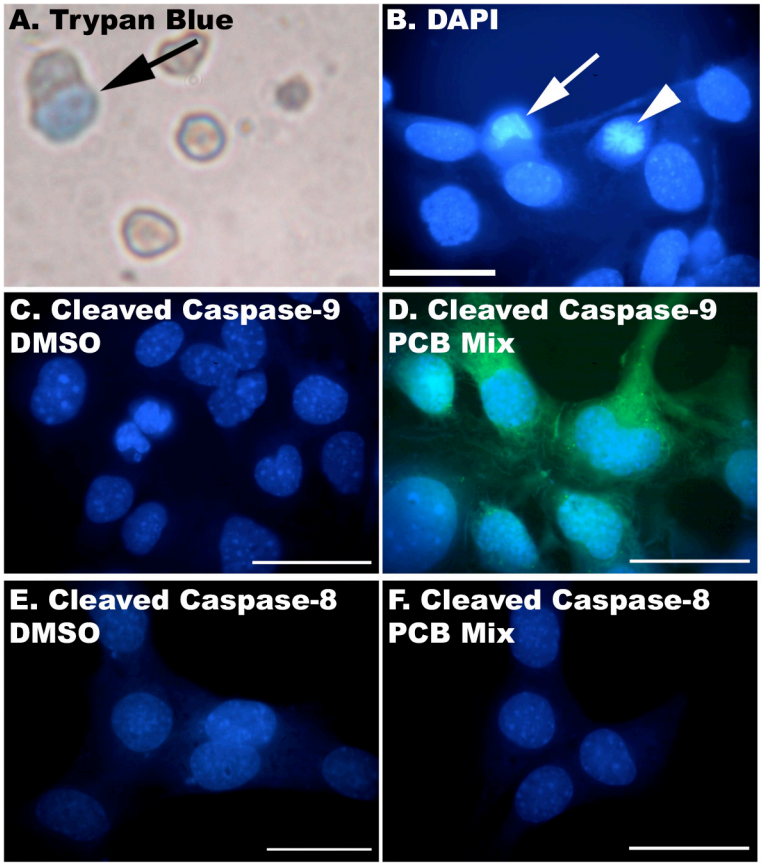


Figure 3.4. Fluorescence assays of markers of cell death are shown. (A) Trypan Blue staining of GT1-7 cells is shown for a representative PCB118 treated culture. Normal cells appear unstained and necrotic cells (arrow) appear blue. (B) DAPI staining of GT1-7 nuclei is shown in a representative PCB74 treated culture. The arrow shows a condensed apoptotic nucleus, and the arrowhead shows a mitotic (non-apoptotic) nucleus. Scale bar = 10 mm (panels A and B). (C and D) Cleaved caspase-9 immunofluorescence is shown for the 4 hour timepoint for a representative control (C) or PCB Mix (D) culture. Results for individual PCBs and the PCB Mix were similar, and all PCBs activated cleaved caspase-9 immunofluorescence. (E and F) Cleaved caspase-8 immunofluorescence is shown for the 4 hour timepoint for a representative control (C) or PCB Mix (D) culture. Results for individual PCBs were indistinguishable from the PCB Mix (not shown). Caspase-8 immunofluorescence was not detectable in any of the PCB- or vehicle-treated cultures. Scale bar = 10 mm (panels C – F).

Figure 3.5: PCBs Induce Necrotic Cell Death

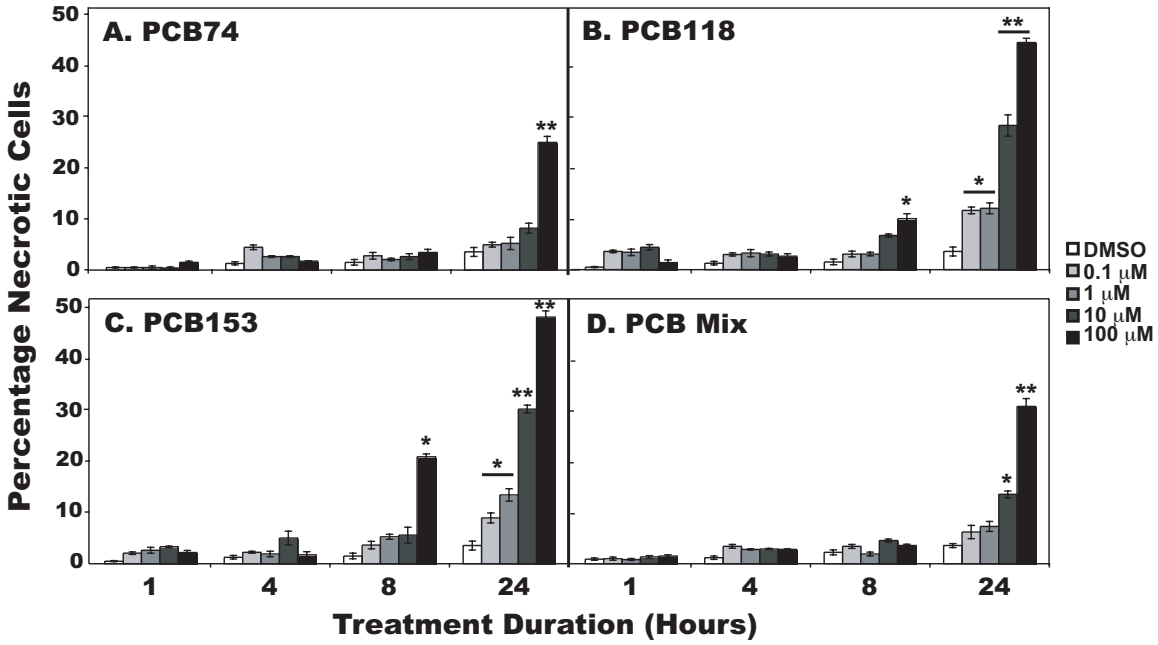


Figure 3.5. The effects of (A) PCB74, (B) PCB118, (C) PCB153, and (D) PCB Mix treatment upon GT1-7 trypan blue staining (necrotic cell death). Cells were incubated in absence (DMSO) or presence of PCB concentrations (0.1, 1, 10, or 100 μM) for different durations (1, 4, 8, 24 hr). Results are expressed as a percentage of blue stained cells characteristic of necrotic cells ± S.E.M for 4 triplicates of each experimental condition. We observed a slight but significant increase in necrotic cell death after 8 h treatment with the highest dose PCB118 or PCB153. After 24 h treatment, the highest dose PCB74 and two highest doses of PCB118 and PCB153 caused an extensive increase in necrotic cell death.

Figure 3.6: PCBs Induce Apoptotic Cell Death

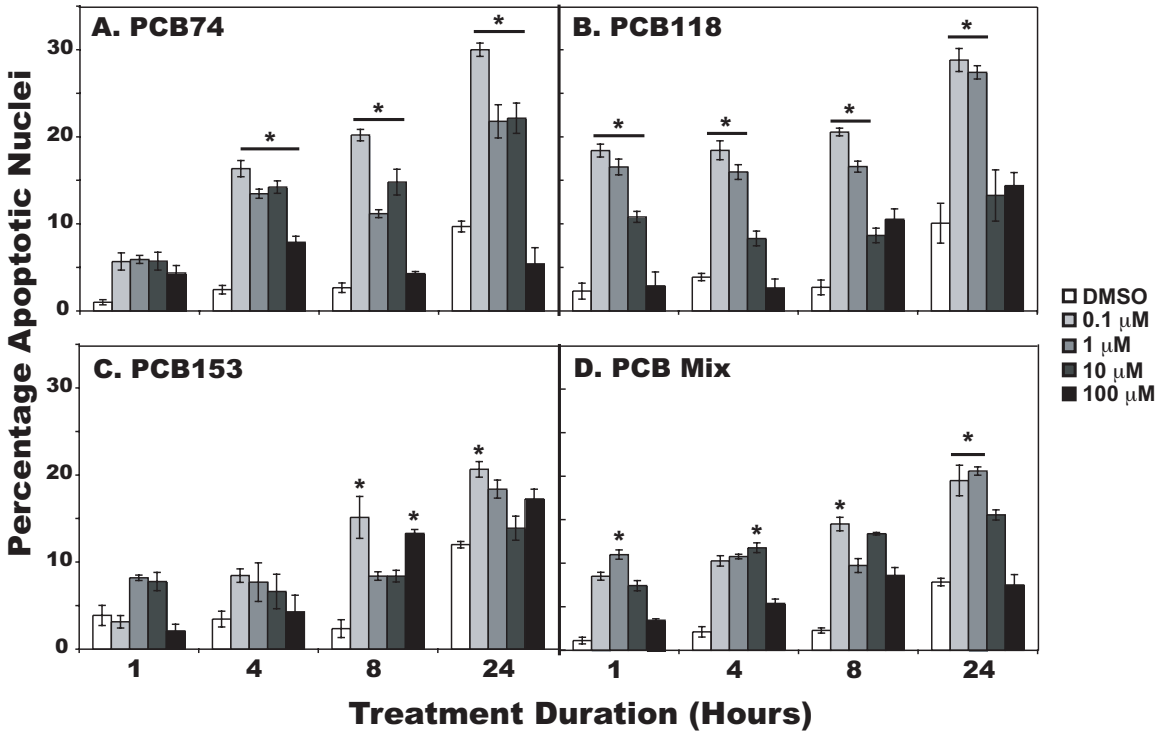


Figure 3.6. The effects of (A) PCB74, (B) PCB118, (C) PCB153, and (D) PCB Mix on GT1-7 apoptotic cell death. Cells were incubated in absence (DMSO) or presence of PCB concentrations (0.1, 1, 10, or 100 μM) for different durations (1, 4, 8, 24 hr). Results are expressed as a percentage pyknotic DAPI stained cells characteristic of apoptotic cells ± S.E.M for 4 triplicates of each experimental condition. We observed a significant increase in apoptotic cell death as early as 1 hr treatment with lower doses (0.1 or 1 μM) PCB74, PCB118, or PCB Mix. After 24 h treatment, all PCBs caused a significant increase in apoptotic cell death.

Figure 3.7: PCBs Activate Caspase 3/7

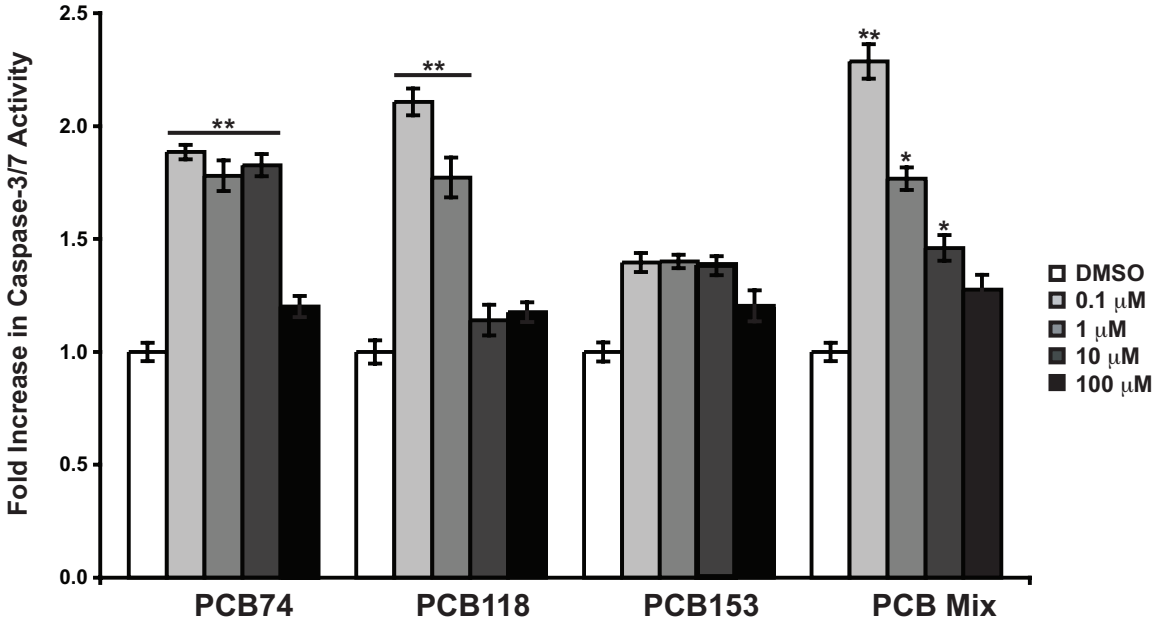


Figure 3.7. The effects of (A) PCB74, (B) PCB118, (C) PCB153, or (D) PCB Mix on caspase-3/7 activation. Cells were incubated in absence (DMSO) or presence of PCB concentrations (0.1, 1, 10, or 100 μM) for four hours. Results are expressed as fold increase of caspase-3/7 activation compared to control cells ± S.E.M for 4 triplicates of each experimental condition. Treatment with PCB74, PCB118 or the PCB Mix resulted in activation of caspase-3/7, while PCB153 did not significantly affect caspase-3/7 activity. *$p < 0.05$, **$p < 0.005$ vs. vehicle (DMSO) at 4 hr.

DISCUSSION

The current study investigated effects of three PCBs, each representing a member of different PCB classes, on GnRH release from the GT1-7 cell line, and on neuroendocrine toxicity due to apoptotic and necrotic cell death. Our results show that PCB74 (coplanar), PCB118 (dioxin-like coplanar), and PCB153 (non-coplanar), significantly affected both GnRH peptide levels and cell survival in the hypothalamic GT1-7 cell line. In general, individual PCBs had an overall stimulatory effect on GnRH peptide levels at early time points and lower concentrations, and inhibitory effects on GnRH peptide at the later time points and higher concentrations. Treatment with PCBs also affected cell survival, resulting in a decrease in viability of up to 60% in GT1-7 cells treated with PCBs for 24 hours. Moreover, the three congeners induced both necrotic and apoptotic cell death, as indicated by cell morphology and DNA fragmentation. Although there were some small differences, for the most part, lower concentrations of PCBs induced apoptosis, while higher concentrations caused necrotic cell death. Thus, PCBs caused both endocrine disrupting and neurotoxic effects in the GT1-7 cell line. Further, effects of a mixture of the three PCBs were not substantially different from those of the individual congeners. Therefore, at the dosages used in the current study, little additivity or synergism could be ascertained.

PCB effects on GnRH peptide concentrations

The three classes of PCB congeners altered GnRH peptide levels in GT1-7 cell media. Effects of PCB74, PCB118, and the PCB Mix were similar: in general, GnRH peptide concentrations were elevated at intermediate to high dosages at the 4 hour timepoint, and lower at the 8- and 24-hour timepoints compared to DMSO controls. PCB153 was slightly different in its effects, as it was the only PCB to be associated with a change at the 1-hour timepoint (increased GnRH peptide at the lowest and highest concentration). By 24 hours, GnRH peptide levels were lower in PCB153 treated cells at the three highest concentrations.

Overall, these studies on GnRH peptide suggest that PCBs may initially stimulate but then inhibit GnRH synthesis and/or secretion. The mechanisms by which GnRH peptide levels are altered by PCBs across time are likely attributable to changes in: (i) GnRH decapeptide biosynthesis; (ii) the release of GnRH from GT1-7 cells, independently of changes in biosynthesis; (iii) GnRH peptide degradation; and/or (iv) number of viable GT1-7 cells. The GnRH measured in culture media may have been secreted from live GT1-7 cells, but it is also possible that cellular stores of the decapeptide were released upon membrane disintegration from dying cells. Here, the initial increase in GnRH does not coincide with a loss in cell viability, making it unlikely that the GnRH levels measured can be attributed to dying cells dumping their stores of the decapeptide. At the earlier time points, the elevated GnRH levels are more likely to

be due to a stimulatory action of PCBs on release of peptide stores. Because PCBs have been shown to act through several neurotransmitter receptor systems, some of which can affect GnRH release and whose receptors are expressed on GT1 cells (e.g., serotonin, dopamine, norepinephrine – (Khan and Thomas, 1997; Mariussen *et al*., 1999; Mariussen and Fonnum, 2001), the activation of these neurotransmitter receptors may have the transient action of causing a release of GnRH. However, the number of viable GT1-7 cells probably plays a role in the longer timepoints, as GT1-7 cell viability was decreased, and apoptosis and necrosis increased, over time (see below).

At the molecular level, the actions of PCBs on endocrine systems are thought to be mediated, at least in part, by nuclear estrogen receptors. Therefore, we tested PCBs together with ICI 182,780, a nuclear estrogen receptor antagonist that can act at both estrogen receptors α and β (Wijayaratne *et al*., 1999), and which has previously been shown to block some effects of estradiol in GT1-7 cells (Gore *et al*., 2002) and other experimental models (Lee *et al*., 1999). In the current study, ICI 182,780 caused a partial reversal of the effects of the individual PCBs or their mixture on GnRH peptide secretion, suggesting potential involvement of an estrogen receptor-dependent mechanism, but also indicating that other mechanisms must be involved. We were surprised by the consistency of this result across the three classes of PCBs, as the three dimensional structure, degree of chlorination, and dose of a PCB congener contributes not only to its toxicity, but also to its interaction with steroid hormone receptors. Congeners that are lightly chlorinated tend to be more estrogenic, whereas the heavily chlorinated congeners may act as a weak estrogen receptor agonist or antagonist in a dose-dependent manner

(Safe, 1994; Bonefeld-Jorgensen *et al*., 2001). It is possible that a wider range of concentrations and duration of treatment may be necessary to better differentiate the role of the estrogen receptor in mediating these processes, since current experiments were performed at the 24 hour timepoint at a single concentration (1 mM) of PCBs.

In addition, it should be stressed that these PCBs may also act through other non-nuclear estrogen receptors, or through non-estrogenic pathways altogether. In primate GnRH neurons, ICI 182,780 does not block the estradiol-stimulated release of GnRH that is mediated through GPR30, the membrane G-protein coupled estrogen receptor (Noel *et al*., 2009). In fact, ICI 182,780 has been described as an agonist at the orphan receptor GPR30 (Thomas *et al*., 2005). However, because PCBs have a relatively low binding affinity for GPR30 *in vitro* (Thomas and Dong, 2006), PCB action on other non-estrogen membrane receptors, e.g. neurotransmitter receptors (Seegal *et al*., 1991a; Khan and Thomas, 1997; Mariussen and Fonnum, 2001) may also account for some of the observed effects. Future experiments investigating the potential role of GPR30 or other membrane receptors will provide a more complete understanding of the mechanisms underlying the observed effects of PCBs on GnRH release.

PCB effects on apoptotic and necrotic cell death

Previous studies have shown that PCBs can be neurotoxic, and that the mechanism can involve either or both apoptotic and necrotic cell death. Using primary fetal cortical neuron cultures, Sanchez-Alonso and colleagues (2003) found that neuronal

apoptosis reached 30 – 50% within 4 hours of incubation with 100 μM PCB77 (a dioxin-like, coplanar PCB), which was also more cytotoxic than the non-coplanar congener PCB153. These effects may be tissue-specific. Howard et al. (2003) found that the non-coplanar PCB47 (1 μM) induces apoptosis in primary cultured hippocampal neurons, but not cortical neurons. In the same study, the dioxin-like coplanar PCB77 (1 μM) had no effect on apoptosis in either cell type (Howard *et al*., 2003), a discrepancy which may be attributable to the lower dose used. A recent study by Ndountse and Chan (2008) found that the non-coplanar congener PCB99 had greater potency for apoptotic cell death in human neuroblastoma cells compared to a dioxin-like coplanar PCB126 and or the PCB mixture, Aroclor 1254 (Ndountse and Chan, 2009). In the same study, necrotic cell death was also observed, and was attributed to the secondary necrosis that follows apoptotic cell death. Necrosis may also be the primary form of cell death caused by PCBs. For instance, a study by Johansson and colleagues (2006) found that both PCB126 and PCB153 induce necrotic cell death in AtT20 pituitary cells, although PCB126 was more potent in this regard (Johansson *et al*., 2006). Merritt and Foran (2007) found that PCB153 caused necrotic cell death in two human glioblastoma cell lines at doses as low as 5000 nM (Merritt and Foran, 2007). Similarly, Mariussen et al. (2002) found that Aroclors 1242 and 1254 and PCB153, but not PCB126, caused extensive cell death in cultured rat cerebellar granule cells after 24 h treatment duration with doses as low as 12.5 μM (Mariussen *et al*., 2002). Together, this literature suggests neurotoxic effects of PCBs, particularly due to a combination of apoptotic and necrotic mechanism. However,

prior to the current study, to our knowledge this question of whether and how PCBs may exert such effects in a neuroendocrine cell system has not been addressed.

To this end, in the present study, we quantified cellular viability in response to individual PCBs or a mix, and determined whether this was due to apoptosis or necrosis. First, we found that the individual PCBs diminished cell viability, with PCB74 and PCB118 having such actions by the first hour of treatment. PCB153 was associated with a diminution of viability beginning at the fourth hour of treatment. The PCB Mix was, surprisingly, the least likely to cause diminished GT1-7 cell viability. Second, we ascertained effects of PCBs on necrosis. This mechanism for cell death was not observed until 8 hours (PCB118, PCB153, both at the highest concentrations) to 24 hours (all PCBs, particularly at the higher concentrations). Third, effects of PCBs on apoptosis were evaluated, with PCB74 and PCB118 having the greater potencies for induction of apoptotic cell death, especially at low to intermediate concentrations. This latter result with PCB74 and PCB118 is consistent with the decreased viability detected in cells treated with these two congeners. Differences in results among the PCBs suggest that the relative neurotoxic potency of individual PCB congeners for induction of neuronal cell death is dependent upon PCB structure.

As a whole, our observations also show that lower doses of PCBs induce apoptosis, and higher doses induce necrosis (c.f. Figures 6 and 7). This result is consistent with other studies establishing a dose-dependent relationship between apoptosis and necrosis in neuronal cells exposed to toxicants (Bonfoco *et al*., 1995). However, the level of apoptotic cell death induced by PCBs in the present study is not as extensive as

that observed in previous studies, a discrepancy likely due to differences in cell lines and methodology. In the dose range used in the current study, the loss of cell viability after 4 hours of treatment can be attributed to the induction of apoptosis. In contrast to previous studies, here we found that the three classes of PCBs had similar potencies for induction of apoptosis at the dose range studied. At later timepoints, the loss of cell viability is due primarily to the induction of necrotic cell death. It is possible that some of the necrotic cells observed were due to the secondary necrosis that follows apoptosis. We also observed some apoptotic and necrotic cells after 24 h in the vehicle treatments alone. Serum starvation alone induces apoptosis over time, including in the GT1-7 cell line (Srinivasan *et al*., 1996). However, differences between vehicle and PCB treatments indicate that the PCBs themselves are inducing these responses.

Apoptosis proceeds via the activation of caspases, a family of cysteine aspartate specific proteases (Nunez *et al*., 1998). Stimulation of membrane death receptors activates caspase-8 and initiates the extrinsic apoptotic pathway, while release of mitochondrial signaling factors and subsequent cleavage of caspase-9 activates the instrinsic apoptotic pathway. Regardless of the pathway initially activated, caspase-3 is widely regarded as the effector caspase, and is activated by both death receptor- and mitochondrial-mediated apoptosis. Once caspase-3 is activated by cleavage, the cell reaches a "point of no return" in the development of apoptosis. Thus, caspase-3 activation (assayed in concert with caspase-7) provides a useful marker of early apoptosis.

The present study demonstrates that coplanar and dioxin-like coplanar PCB congeners (PCB74, PCB118) and their mixture activate caspase-3, consistent with

induction of apoptotic cell death. Moreover, we observed the activation of caspase-9 by all of the PCB treatments, which is associated with the mitochondrial-mediated intrinsic pathway of apoptosis. Our results are consistent with other studies that have demonstrated activation of caspase-9 following exposure to a non-coplanar (Ghosh *et al*., 2007) and dioxin-like coplanar (Hsu *et al*., 2007) PCB. By contrast, caspase-8 was not activated by any PCB treatment, suggesting that this mechanism is not in play in the GT1-7 cell neurotoxicity.

Environmental endocrine disruption by PCBs

Developmental apoptosis, a process that is regulated by activation of estrogen receptors (McCarthy, 2008), plays a critical role in sexual differentiation of the hypothalamus and is necessary for normal maturational processes involved in the attainment and maintenance of reproductive function. PCBs may disrupt developmental apoptosis through actions on estrogen receptors as well as by other mechanisms of action in the nervous system. GT1-7 cells were originally isolated from developing GnRH neurons that have both mature neuronal properties but are not post-mitotic due to transformation with the SV-40 large T antigen (Mellon et al., 1990). Other exogenous compounds have effects on the developing brain *in vivo* at a range of micromolar concentrations similar to the one used in this study. For instance, a recent study found that the phytoestrogen genistein affects developmental apoptosis in the brain of zebrafish embryos at micromolar levels (Sassi-Messai *et al*., 2009). It is reasonable to postulate that

PCB-induced apoptosis or necrosis may be related to the neurotoxic effect of these EDCs on the reproductive axis, and that different molecular mechanisms may operate in the induction of cell death, depending on the structure of the PCB congeners, the neuronal population studied, and the developmental stage. Therefore, our results have considerable relevance to exposures of wildlife, humans and laboratory animals to PCBs, particularly when exposure occurs during key developmental life stages such as the period of sexual differentiation.

Given that PCB74, PCB118, and PCB153 are congeners frequently found in environmental (Lake *et al*., 1995), wildlife (Kunisue *et al*., 2003), and human lipid and serum samples (Larsen *et al*., 1994; Lanting *et al*., 1998; Freels *et al*., 2007; Park *et al*., 2007), results from this study are relevant for ecological exposures. As the human body burden of the three PCB congeners used in this study varies considerably by individual and tissue, and it is difficult to extrapolate from *in vitro* to *in vivo*, the doses in our study were chosen to approximate human levels. Because PCBs differentially accumulate into microenvironments in living tissues, with levels reaching approximately 50 ppb in neonatal human brains (Lanting *et al*., 1998), developing neurons may be exposed to micromolar concentrations of these toxicants. This study establishes the role of PCBs in the induction of apoptosis in GnRH GT1-7 cells as a model for developing hypothalamic neurons. Future experiments in animal models will apply the present observations in GT1-7 cells on the effects of PCB exposure on the developing GnRH neurosecretory system.

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CHAPTER 4: EFFECTS OF PRENATAL PCB EXPOSURE ON NEUROENDOCRINE DEVELOPMENT IN THE NEONATAL RAT

The text in this section is modified from the article "Prenatal PCBs Disrupt Early Neuroendocrine Development of the Rat Hypothalamus," Dickerson SM, Cunningham SL, and Gore AC, (*Toxicology and Applied Pharmacology*, submitted; 2010).

ABSTRACT

Neonatal exposure to endocrine disrupting chemicals (EDCs) such as polychlorinated biphenyls (PCBs) can interfere with hormone sensitive developmental processes, including brain sexual differentiation, and permanently alter reproductive physiology in adulthood. We tested the hypothesis that the disruption of these processes by gestational PCB exposure would be detectable as early as the day after birth (postnatal day (P) 1). Fetuses were exposed through injection of pregnant Sprague-Dawley rats on gestational days 16 and 18 with DMSO (vehicle), Aroclor 1221 (A1221, an estrogenic PCB mix), a reconstituted PCB mixture of PCB138, PCB153 and PCB180 (the three most prevalent congeners found in human samples), or estradiol benzoate (EB), an estrogenic control. On P1, litters were assessed for number of pups and sex ratio, and each pup was measured for anogenital distance (AGD) and body weight. Then, pups were euthanized to enable immunohistochemistry of estrogen receptor α (ERα) and TUNEL labeling, or gene expression measurements of 48 selected genes on a Taqman PCR array platform. AGD in P1 females was reduced (hyperfeminized), whereas male AGD was

increased (hypermasculinized), by prenatal EDC treatments. Treatment with EB or A1221 had a sex-specific effect on developmental apoptosis in the neonatal anteroventral periventricular nucleus (AVPV): exposed females had increased numbers of apoptotic nuclei, with no effect in males. ERα immunoreactive cell number was increased by EB treatment in both males and females, while PCBs had no effect. PCR analysis identified brain-derived neurotrophic factor, GABAB receptors-1 and -2, IGF-1, Kisspeptin receptor, NMDA receptor subunits NR2b and NR2c, prodynorphin, and TGFα mRNAs as significantly affected in P1 males or females. Collectively, these results suggest that the disrupted sexual differentiation of the POA can be detected as early as the day after birth, effects that may underlie the adult reproductive phenotype.

INTRODUCTION

Exposure to environmental endocrine disrupting chemicals (EDCs) during critical developmental periods, particularly late gestation and infancy, are consistently linked to impairments in homeostatic, endocrine, and neurobiological processes in adulthood (Dickerson and Gore, 2007). Amid concerns that chronic low-dose exposures to EDCs may be contributing to a decline in fertility in humans (Diamanti-Kandarakis *et al*., 2009), recent interest has turned to elucidating how reproductive neuroendocrine systems may be perturbed by EDC exposures during early critical life stages. As the hypothalamic control of reproduction develops in a sexually dimorphic manner due to sex differences in gonadal steroid hormone actions in the brain, it is plausible to hypothesize that some of

the links between perinatal EDCs and the diminution in reproductive competency may be due at least in part to reprogramming of the neonatal hypothalamus by these compounds.

Sexual differentiation of the hypothalamus of rodents occurs during the third trimester of gestation through the early postnatal period. During these life stages, the number and phenotype of neurons that arise in the sexually dimorphic nuclei of the hypothalamus are sculpted by a number of neurodevelopmental processes, including programmed cell death, called apoptosis (Davis *et al*., 1996a; Yoshida *et al*., 2000). Whereas the female rodent brain develops in relatively low levels of gonadal hormones, the male brain is awash in estradiol locally aromatized from the testicular hormone testosterone, and differences in these neural exposures (Bakker and Brock, 2010) have profound effects on the numbers of cells that ultimately survive in a region-specific manner.

Importantly, there are links between steroid hormone exposures and apoptosis. Depending upon the brain region, ligand-bound estrogen receptors (primarily ERα) may bind nuclear response elements that promote transcription of factors that either stimulate or inhibit developmental apoptosis (McCarthy, 2008). This point is exemplified by developmental differences in apoptosis in the neonatal anteroventral periventricular nucleus (AVPV), a region that differs in size and cellular phenotype between males and females (Clarkson and Herbison, 2006) and is postulated to mediate estradiol positive feedback onto the GnRH/LH surge in females (but not males, which do not have this surge). In the developing AVPV, estradiol stimulates apoptosis during the early postnatal period (Murakami and Arai, 1989; Sumida *et al*., 1993; Waters and Simerly, 2009),

contributing to the development of a smaller AVPV in adult males (Davis *et al*., 1996b), and presumably, differences in reproductive physiology and behavior. Another brain region under investigation in this study is the medial preoptic area (MPN), a region important for adult reproductive behaviors (Wu *et al*., 2009a; Wu and Gore, 2010), sexually dimorphic in size (Madeira *et al*., 1999), and abundant in ERs (Chakraborty *et al*., 2003). Evidence that EDCs can act upon ERα and other steroid hormone receptors in the nervous system suggests that they may perturb developmental apoptosis in the AVPV and MPN, a hypothesis tested in this current study.

Our laboratory has been using a class of compounds known as polychlorinated biphenyls (PCBs), a family of persistent chemicals once used widely for industrial applications, as a model for neuroendocrine disruptors (Salama *et al*., 2003; Steinberg *et al*., 2007; Steinberg *et al*., 2008; Dickerson *et al*., 2009). Although banned for decades, PCBs are still detectable in the body burden of most humans (Gladen *et al*., 2003). EDCs rarely exert their effects by a single mechanism, and PCBs have been shown to be weakly estrogenic (Mortensen and Arukwe, 2008; Nomiyama *et al*., 2010), as well as anti-estrogenic and anti-androgenic, among other mechanisms (Bonefeld-Jorgensen *et al*., 2001). We utilized two PCB mixtures that differ in their half-life, degree of chlorination, and chemical properties. Aroclor 1221 (A1221; an estrogenic PCB) is a technical mixture once used commercially, and is comprised of lightly chlorinated congeners with a half-life on the order of days (Matthews and Anderson, 1975). We also used a reconstituted mixture of the three most prevalent congeners detected in human and wildlife samples: PCB138, PCB153, and PCB180, which are more heavily chlorinated and have a half-life

on the order of years (Milbrath *et al*., 2009; Seegal *et al*., 2010). *In vitro* studies have shown that these compounds interact with steroid hormone receptors including ERα at low doses (Bonefeld-Jorgensen *et al*., 2001). Therefore, we tested the hypotheses that prenatal EDCs would affect developmental apoptosis and expression of ERα in a sexually dimorphic manner, an effect that could be detectable as early as postnatal day (P) 1. We also used a 48-gene PCR-based array to identify novel gene expression targets of developmental PCB exposure on the P1 hypothalamus.

METHODS

Animals and perinatal treatment

All protocols on rats were carried out following guidelines from the National Institute of Health Guide for Care and Use of Laboratory Animals, and performed following protocols approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Dams for this study (Harlan Sprague–Dawley Inc.; Houston, Texas; Stock/Strain: Hsd:Sprague–Dawley®™ SD®™) were those described in a sister study on effects of prenatal PCBs on the adult hypothalamus (Dickerson *et al*., 2011). Rats were housed individually under standard husbandry [12:12 partially reversed light cycled (lights on 2300 h, lights off 1100 h)] and fed low-phytoestrogen Harlan-Teklad 2019 Global Diet *ad libitum* for at least 3 weeks prior to mating. Rats were handled daily to minimize stress.

Dams were mated with sexually experienced male rats (males randomly rotated with females), and the day of sperm positive vaginal smears was termed embryonic day (E) 1. The pregnant rats (n=10-12 per treatment group) were randomly assigned to one of four treatment groups and injected i.p. on E16 and E18 with one of the following: 0.1 ml of vehicle (DMSO 99.5%, Sigma, #D4540, Lot# 037K07663); 50 μg/kg estradiol benzoate (Sigma, #E8515, Lot# 125K1029, serving as an estrogenic positive control); 1 mg/kg Aroclor 1221 (AccuStandard, #C-221N, Lot# 083-166; dose based on our published work showing effects on reproductive function in female rats (Steinberg *et al*., 2007; Steinberg *et al*., 2008); or 1 mg/kg reconstituted PCB mixture (referred to as PCB Mix). Rationale for the PCB Mix that these are the three most prevalent PCB congeners found in mammalian tissue samples (Gladen *et al*., 2003; Bentzen *et al*., 2008): PCB138 (2,2',3,4,4',5'-Hexachlorobiphenyl; AccuStandard, #C138N, Lot# 082704MS-AC), PCB153 (2,2',4,4',5,5'-Hexachlorobiphenyl; AccuStandard, #C153N, Lot# 111804AG-AC), and PCB180 (2,2',3,4,4',5,5'- Heptachlorobiphenyl; AccuStandard, #C180N, Lot# 013004MT-AC) at equimolar concentration. It should be noted that they are non-coplanar and do not bind the aryl hydrocarbon receptor (Van den Berg *et al*., 1998). The dose, age and route of exposure were based on the literature on detectable levels of PCBs in humans (Lanting *et al*., 1998; Lackmann, 2002), the timing of brain sexual differentiation in rats (Murakami and Arai, 1989; Rhees *et al*., 1990a), and for consistency with other neuroendocrine studies including our own (Chung and Clemens, 1999; Chung *et al*., 2001; Gore *et al*., 2002; Salama *et al*., 2003; Woodhouse and Cooke, 2004; Steinberg *et al*., 2008). Although we have not measured PCB content in the offspring, we have

previously predicted that pups were exposed to approximately 2 μg/kg total PCBs for both the A1221 treatment group and the PCB Mix treatment group (Takagi *et al*., 1986; Steinberg *et al*., 2007; Steinberg *et al*., 2008). The day after birth, P1, the numbers of live and dead offspring were counted, and sex ratio was determined. Anogenital distance (AGD) was measured using a digital microcaliper (Marois, 1968; Steinberg *et al*., 2008), and the ratio of AGD to the cube root of body weight (AGD index) was calculated to evaluate AGD (Marois, 1968; Gallavan *et al*., 1999).

Tissue collection

The offspring were divided into two groups and either sacrificed at P1 between 0900 – 1000 h to evaluate experimental endpoints reported herein, or allowed to mature for a study reported elsewhere (Dickerson *et al*., 2011). One male and female F1 rat per litter was euthanized for protein and apoptosis studies (N=8 per sex, each from different litters), and 1 male and female per litter was utilized for gene expression studies (N=6 per sex, each from different litters). For immunohistochemistry studies, pups were deeply anesthetized with 0.05 ml ketamine (100 mg/ml) and 0.05 ml of xylazine (20 mg/ml), and trans-cardially perfused with 0.9% saline (5 ml) at a rate of 5 ml/min, followed by 4% paraformaldehyde (50 ml). The brains were removed and postfixed overnight in 4% paraformaldehyde, and then transferred into PBS with 0.2% sodium azide. A vibrating microtome (Leica VT 1000S, Leica Microsystems, Nussloch, Germany) was used to cut 50 μm-thick sections that were stored in PBS with 0.2% sodium azide at 4 °C until use.

For gene expression analyses, animals were rapidly euthanized by decapitation. Brains were quickly removed and the preoptic area-anterior hypothalamus (POA), which contains the AVPV and medial preoptic nucleus (MPN), was dissected on ice, snap frozen (Dickerson *et al*., 2008) and stored at -80 C until RNA extraction. Terminal trunk blood samples were collected and centrifuged at 5000 x g for five minutes to separate serum, and serum was stored at -80°C until steroid hormone analysis. Because EDC treatment can drastically affect AGD of treated animals, the sex of each animal was confirmed via visualization of the uteri or testes at the time of euthanasia.

Immunohistochemistry for ER alpha

Hypothalamic tissues were recoded for tissue processing and analyses so that the experimenter was blind to treatment group and sex. For each neonatal F1 animal, two AVPV tissues and four MPN tissues per rat were immunolabeled for ERα. Although the number of sections was too great to process in a single run, animals from each sex and treatment group were equally represented in every run. Sections were rinsed in PBS (Phosphate-buffered saline, pH = 7.4) at room temperature on a shaker, followed by treatment with 3:1 methanol: 3% H_2O_2 for 20 minutes at room temperature to eliminate endogenous peroxide activity. Sections were then washed, and incubated in the rabbit polyclonal anti-ERα antibody C1355 (1:10,000; Upstate Biotechnology, Waltham, MA) with 10% normal goat serum (NGS) and 0.1% Triton-X for 72 hours at 4°C on a shaker. This antibody was generated against the last 15 amino acids of ERα, which has no homology to the corresponding region of ERβ. The sections were then washed and

incubated in 5% normal goat serum and secondary antibody (biotinylated goat anti-rabbit immunoglobulin (Ig)G, 1:600; BA-1000; Vector Laboratories) for one hour, then subjected to peroxidase reaction with nickel-enhanced DAB. Sections were mounted on gelatin-subbed slides, dried, dehydrated in a graded alcohol series, counterstained with methyl green, and coverslipped with DPX (44581; Fluka, Steinheim, Germany). Controls were also run with the primary antibody omitted, and no specific binding was observed.

Because ERα labeling was sparse and highly variable in the AVPV, stereological analysis for this region was not possible. Quantification of ERα in the MPN was performed using unbiased stereological analysis according to methods described in detail previously (Chakraborty *et al*., 2003; Chakraborty *et al*., 2005). A wet-mount of fresh tissue showed that average tissue thickness was 50.6 μm. The MPN region was identified in Nissl-stained sections by comparing anatomical landmarks to an atlas of the developing rat brain (Ashwell, 2008). Contours were drawn around the MPN at low magnification (10× objective) using an Olympus BX-61 microscope. A buffer zone at the top and bottom of sections was set at 3 μm for all experimental stereology. For each rat, the regional volume was extrapolated based on the contours and tissue thickness (Volume = regional area × thickness). The Stereo Investigator® software (MicroBrightField, Williston, VT) randomly placed 75 μm × 120 μm grids ("dissector frames") within the MPN contour. Within these dissector frames, the DAB-stained ERα-labeled nuclei were counted within a 45 μm × 45 μm counting frame for the MPN ("optical dissectors"). Based on these parameters, the number and density (# immunoreactive cells/volume of each nucleus) of ERα-immunoreactive (ERα-ir) nuclei

falling within the region was quantified. The coefficients of error (Cruz-Orive/Geiser) and variation of the estimates were calculated as described previously (Schmitz and Hof, 2000). Photomicrographs were taken to produce the figures, and images subjected to only minor adjustments of contrast using Adobe Photoshop CS4 (Adobe, San Jose, CA), with any adjustments were applied equally to tissues from rats of different treatment groups.

Detection of apoptosis

To stain the nucleosomal DNA fragments in apoptotic cells, we used a modification of the TUNEL method described by Bessert and Skoff (Bessert and Skoff, 1999). All reagents were supplied by the Fluorescein-FragEL DNA Fragmentation Detection Kit (EMD Chemicals Inc., Gibbstown, NJ) and applied according to the kit protocol at room temperature (RT) unless otherwise noted. In brief, tissues were pretreated with sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) at 60°C for 15 min, then washed in 0.1 M PBS, pH 7.4, incubated in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for 15 min, and washed in 0.1 M PBS. The sections were then treated with 20 μg/ml proteinase K in 10 mM Tris pH 8 for 15 min and washed in PBS. The sections were incubated in equilibration buffer for 30 min, followed by incubation in labeling reaction mixture in a humidified chamber at 37°C for 1.5 hr. Sections were washed in PBS and mounted onto gelatin subbed slides, and coverslipped with Fluorescein-FragEL™ Mounting Media. Positive controls for the TUNEL assay were generated by pretreatment of sections with DNAse I (Applied Biosystems Inc., Foster City, CA). Negative controls for the TUNEL procedure were

treated in the same manner as the test samples except that the TdT enzyme was omitted from the reaction mixture and was replaced with dH_2O. No labeling was found in the negative controls. Mounted sections were stored in the dark at 4°C until analysis.

A 1:2 series from the AVPV (2 sections total) and 1:2 series from the MPN (4 sections total) were selected from each animal. For apoptosis studies, numbers of labeled cells were counted unilaterally throughout the rostrocaudal extent of the region. Using StereoInvestigator software (MicroBrightField Inc.), contours were drawn for each region at low magnification (10× objective) using anatomical landmarks and a developing rat brain atlas (Ashwell, 2008). Ten counting grids measuring 25 μm X 25 μm per were randomly placed within the left hemisphere for the AVPV and ten counting grids measuring 45 μm X 45 μm were placed on the left side of the third ventricle for the MPN. Total numbers of apoptotic cells were counted for each region at high magnification (100× objective).

Serum Hormone Assays

Because of the small amount of serum obtainable from a P1 rat, serum samples from 2-3 same-sex siblings were pooled for littermates (N=8 pooled samples per sex per assay), and samples run in single testosterone, progesterone, or estradiol RIAs as described previously (Dickerson *et al*., 2011). Briefly, total serum testosterone was determined using the Active® Testosterone coated well EIA kit (Catalog # DSL-10-4000, Lot # 08035-B, Diagnostic Systems Laboratories, Inc., Webster, TX, USA) on duplicate volumes of 50 μl serum. The assay limit of detection was 0.04 ng/ml, and the intra-assay

CV based on duplicate samples for the assay was 2.97%. Progesterone concentrations were determined using the ACTIVE® Progesterone Coated-Tube Radioimmunoassay Kit (Catalog # DSL-3900, Lot # 07076, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), on duplicate volumes of 25 μ1. The sensitivity of the assay was 0.12 ng/ml, and the intra-assay CV was 3.26%. Estradiol concentrations were determined using an ultrasensitive double-antibody RIA kit (Catalog # DSL-4800, Lot # 07076, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), on duplicate volumes of 200μL. The assay limit of detection was 2.2 pg/mL, and the intra-assay coefficient of variability based on duplicate samples was 7.97%. For all assays, a few samples for which the CV between duplicates was 10% or greater were excluded from analysis.

RNA extraction

Messenger RNA from frozen POA dissections was extracted using an in-house double detergent lysis buffer system as described previously (Dickerson *et al*., 2008; Walker *et al*., 2009). In brief, samples were homogenized and the cytoplasmic RNA was treated with proteinase K, followed by extraction with phenol chloroform and precipitation in isopropanol. Genomic DNA contamination was removed using the TURBO DNA-free kit (Applied Biosystems Inc., Cat. No. AM1907, Foster City, CA) according to the manufacturer's protocol. The concentration of resulting cytoplasmic RNA was determined using a Nanodrop (ND-1000, Nanodrop Technologies, Inc., Wilmington, DE).

Taqman low-density arrays (TLDA)

Samples were run as described by our laboratory (Walker *et al*., 2009) on a custom rat neuroendocrine TLDA (Applied Biosystems Inc., Foster City, CA), a panel of 48 candidate neuroendocrine genes. Cytoplasmic RNA (2 μg) was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The resulting cDNA was diluted 1:5 before PCR reactions were performed using Applied Biosystems' Taqman reagents on an ABI 7900 real-time PCR machine using the following parameters: 50 C for 2 min, 94.5 C for 10 min, 45 cycles of 97 C for 30 sec, and 59.7 C for 1 min. Relative expression for each gene was determined using the comparative Ct method (Pfaffl, 2001). Each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (Walker *et al*., 2009), and the data were calibrated to the average change in Ct for the treatment group with the lowest expression.

Statistical Analysis

SPSS statistical software (17.0 for Macintosh, SPSS Inc., Chicago, IL) was used to evaluate the effects of treatment on litter composition, body weight, AGD, serum hormones, AVPV and MPN volume, AVPV and MPN ERα immunoreactivity and apoptosis endpoints. We had independent *a priori* hypotheses for each sex; thus, statistics were performed separately for males and females. Datasets were examined for homogeneity of variance and normality. For datasets that met these criteria, comparisons were made by one-way ANOVA (factor: treatment) followed by Fisher's LSD post-hoc

analysis when indicated by a significant main effect. When variance between treatment groups was unequal, datasets were compared using the nonparametric Kruskal-Wallis test. In all these cases *p*-values < 0.05 were considered statistically significant. For the gene expression data, statistics were conducted using the normalized Ct (δ-Ct) for each sample (before transformation to fold change). Because the TLDA measures expression of 48 genes, a Bonferroni correction was used to set the cut off for significance at $p < 0.001$.

RESULTS

Litter composition, birth weight and anogenital distance

On postnatal P1, litter composition, and birth weight and anogenital distance (AGD) were recorded for all pups in each litter. There was no effect of treatment upon total (DMSO: 13 ± 0.43; EB: 12.3 ± 0.85; A1221: 12.4 ± 1.0; PCB: 10.8 ± 1.3) or live (DMSO: 10.9 ± 0.81; EB: 10.9 ± 0.97; A1221: 11.8 ± 1.0; PCB: 10.2 ± 1.5) pup number per litter, or upon sex ratio (DMSO: 49 ± 3%; EB: 40 ± 3%; A1221: 48 ± 3%; PCB: 48 ± 7%). Likewise, there was no main effect of treatment upon body weight in either sex, although we did observe a trend for increased body weight in females treated with EB or A1221 (**Fig. 4.1A**, $p = 0.062$). A sex-dependent effect of treatment on AGD was noted, with EB-, A1221-, and PCB-treated females having reduced AGD ($p<0.001$) and EB- and A1221-treated males having increased AGD ($p<0.05$) compared to their vehicle counterparts (**Fig. 4.1B**).

Figure 4.1: PCB Effect on Birth Weight and Anogenital Distance

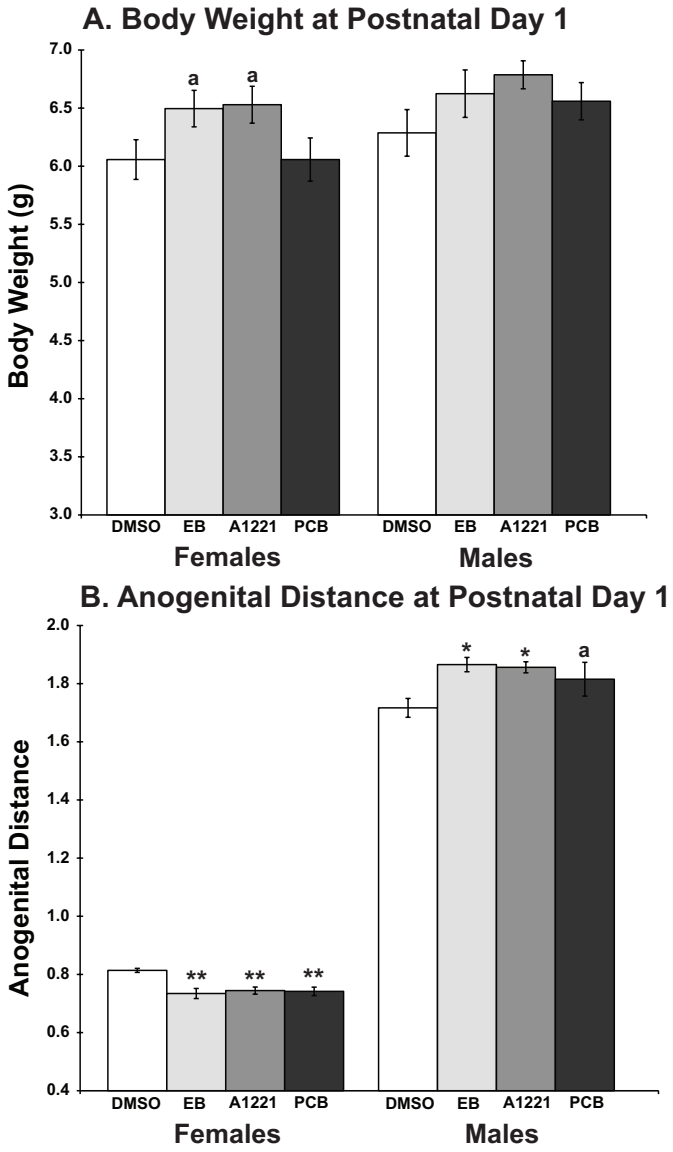

Figure 4.1: Data for body weight (A) and anogenital distance (B) on P1 are shown for female and male rats (mean ± S.E.M). Treatment with estradiol benzoate (EB) or Aroclor 1221 (A1221) caused a non-significant trend for increased body weight in females ($^{a}p=0.06$). For AGD, treatment with EB, A1221, or PCB Mix caused a decrease in female AGD (**$p<0.001$), while EB- or A1221-treatment increased male AGD (*$p<0.05$). Treatment with PCB Mix caused a non-significant trend for increased AGD in males ($^{a}p=0.06$). AGD index is the ratio of AGD (mm) to the cube root of body weight. PCB on the x-axis of this and other figures refers to the PCB Mix group.

Serum hormones

We observed a significant effect of treatment upon serum estradiol in EB-treated females (**Fig. 4.2A**, $p<0.005$), a group with higher estradiol levels than all other groups. A significant main effect of treatment upon serum progesterone was noted in females (**Fig. 4.2B**) but not in males, with A1221- and PCB Mix-treated females having reduced progesterone compared to EB- ($p<0.05$) but not DMSO-treated females. However, no group differed from control in progesterone concentrations. There was a main effect of treatment upon serum testosterone in males (**Fig. 4.2C**, $p<0.001$), but not females, with a reduction observed in EB and PCB-treated males compared to the control and A1221 males.

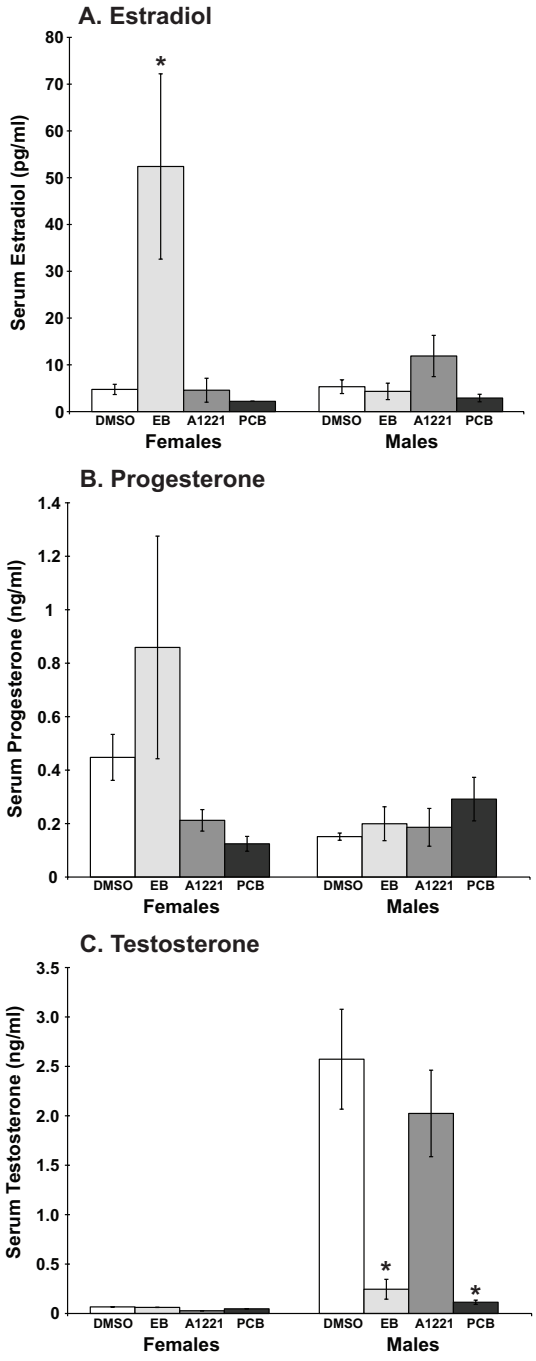

Figure 4.2: Serum levels of estradiol (A), progesterone (B), and testosterone (C) in neonatal F1 animals. For estradiol, EB-treatment caused an increase in estradiol levels in females (*$p<0.005$), while males were unaffected by any treatment. No effect of treatment upon progesterone was observed in males or females. For testosterone, EB- and PCB Mix-treated males had lower serum testosterone compared to DMSO- and A1221-treated males ($p<0.001$ vs. both). N=8 pooled samples per sex per assay. The bars represent the mean ± S.E.M.

Apoptosis in AVPV and MPN

Cells undergoing apoptotic cell death display distinct morphological changes, characterized by cellular shrinkage, nuclear pyknosis, chromatin condensation and membrane blebbing. To ascertain whether prenatal EDC exposure disrupts developmental apoptosis in the neonatal hypothalamus, we used a TUNEL assay and DAPI labeling to visualize apoptotic nuclei in the AVPV and MPN. A representative photomicrograph of TUNEL labeling with DAPI counterstaining is shown for the MPN (**Fig. 4.3A**). In the female AVPV, EB- and A1221-treatment increased the number of apoptotic nuclei (**Fig. 4.3B**, $p<0.001$), while no effect of treatment was observed in males. In contrast, no effect of treatment upon apoptosis was observed in either females or males in the MPN (**Fig. 4.3C**).

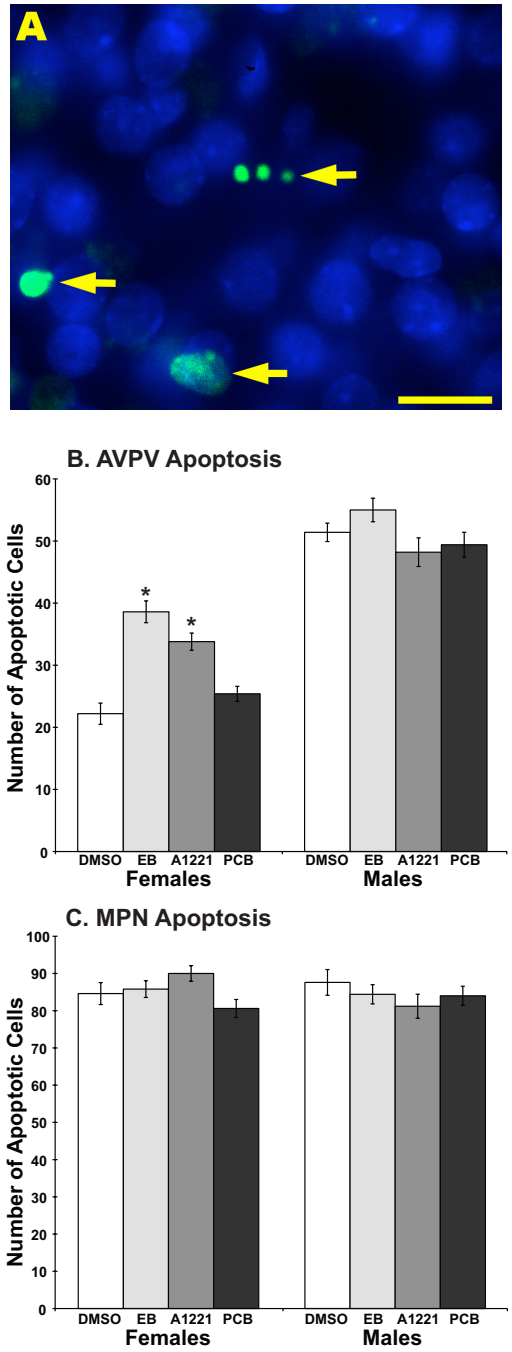

Figure 4.3: A representative photomicrograph shows TUNEL labeling results for early, middle, and late phase apoptosis the MPN (A). TUNEL-positive cells appear bright green (arrows) and DAPI counterstained nuclei appear blue. Scale bar = 25 μm. Also shown are quantification of apoptotic nuclei in the AVPV (B) and MPN (C). In females, EB- and A1221-treated females have a higher incidence of apoptotic nuclei compared to DMSO control (*$p<0.01$), while males were unaffected by treatment. No effect of treatment upon MPN apoptosis was observed in either sex.

ER alpha expression in the AVPV and MPN

In the AVPV, ERα labeling was sparse and highly variable; thus, stereological analysis for this region was not possible. However, qualitative observations suggest that ERα immunoreactivity (ir) in the AVPV is greater in control females compared to control males, and that neither males or females were affected by treatment with EB, A1221, or PCB Mix.

Representative photomicrographs of ERα-ir in the MPN are shown in P1 male and female rats for the four treatments (**Fig. 4.4, A-H**). Stereological cell counting showed a significant main effect of treatment on ERα-ir cell numbers, with EB-treated males and females having significantly more cells than DMSO-, A1221-, and PCB Mix-treated counterparts ($p<0.05$; **Fig. 13A**). No effect of treatment upon MPN regional volume was observed in males or females (**Fig. 13B**). Density of ERα was increased by EB-treatment in MPN of F1 males and females ($p<0.05$; **Fig. 13C**).

Figure 4.4: EDC Effect on ERα Expression in the P1 MPN

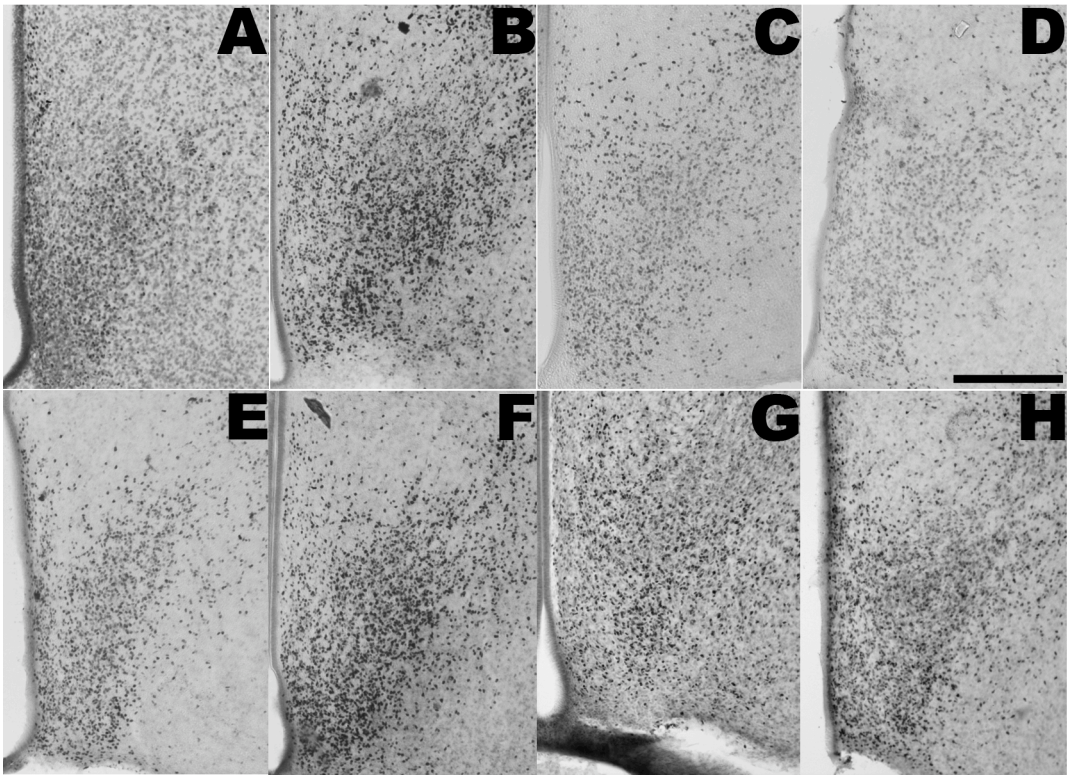

Figure 4.4: Photomicrographs of estrogen receptor alpha (ERα) immunoreactivity in the medial preoptic nucleus (MPN; A-H) of P1 female and male rats prenatally exposed to DMSO vehicle (A,E), EB (B,F), A1221 (C,G), or PCB Mix (D,H). The third ventricle can be seen at the left of each micrograph. Although the images presented here were photographed at low magnification (10X), quantification and analysis of ERα-immunoreactivity was performed at high-power magnification (40X). ERα nuclei are labeled with dark brown nickel-enhanced DAB product. Scale bar = 250 μm.

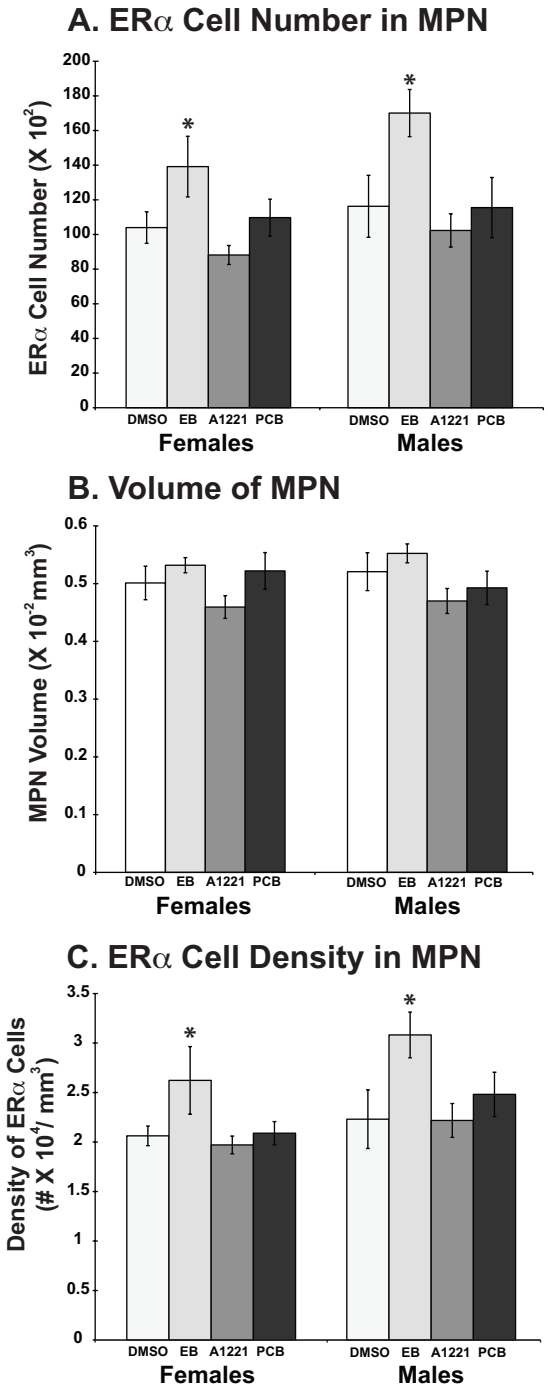

Figure 4.5: Stereologic analysis results for ERα-immunoreactive cell numbers in the MPN (A), regional volume of the MPN (B) and cell density (C) are shown for neonatal P1 rats (n = 6-8 rats per treatment group). Data are the mean ± S.E.M. ERα cell number was significantly increased in EB-treated males and females compared to DMSO control. MPN volume was unaffected by EDC treatment in either sex. Cell density was increased by EB treatment in both sexes. *$p < 0.05$.

Effects of developmental PCB exposure on POA neuroendocrine gene expression

Forty-seven of the candidate genes on the neuroendocrine TLDA were detectable following real-time PCR reactions (**Table 4;** not shown are 18S, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) **"**housekeeping" control genes and the non-detected 3ß-hydroxysteroid dehydrogenase (HSD3b)). As described previously (Walker *et al*., 2009), POA genes were normalized to GAPDH for analyses, as this gene did not vary by treatment. Of the remaining detectable genes, relative gene expression of nine genes was significantly affected by developmental EDC exposure in P1 males or females following the Bonferroni correction (**Fig. 4.6**): brain-derived neurotrophic factor (BDNF), GABAB receptors 1 and 2, IGF-1, kisspeptin receptor (GPR54), NMDA receptor subtypes NR2b and NR2c, prodynorphin, and TGFα.

Table 4. PCB Effects on P1 POA Gene Expression

	Males			Females		
Gene	**EB**	**A1221**	**PCB**	**EB**	**A1221**	**PCB**
Aryl hydrocarbon receptor	0.01 ↓	--	0.05 ↓	--	--	0.009 ↑
Androgen receptor	--	--	--	--	--	--
Aryl hydrocarbon nuclear translocator	--	--	0.06 ↓	0.004 ↑	0.022 ↑	0.026 ↑
Brain-derived neurotrophic factor	**<0.001 ↓**	0.01 ↓	0.01 ↓	--	--	--
Cytochrome P450, 17a1 (17-alpha hydroxylase)	--	--	--	--	--	--
Cytochrome P450, 19a1 (aromatase)	--	--	--	0.037 ↑	--	0.026 ↑
Cytochrome P450, 1b1	-- --	--	0.04 ↑	0.075 ↓	0.062 ↓	0.078 ↑
Estrogen receptor alpha	--	--	--	0.067 ↑	--	0.079 ↑
Estrogen receptor beta	--	--	--	--	--	--
GABA-B receptor 1	0.07 ↓	--	0.028 ↓	**<0.001 ↑**	**<0.001 ↑**	<0.004 ↑
GABA-B receptor 2	0.011 ↓	--	**<0.001 ↑**	--	--	--
Galanin	--	--	--	--	--	--
Gonadotropin-releasing hormone 1	--	--	--	--	--	--
Gonadotropin-releasing hormone receptor	0.037 ↓	--	--	--	--	--
G-protein coupled receptor 30	--	--	--	--	--	--
GluR1	--	--	--	--	--	--
GluR2	0.072 ↓	--	0.042 ↓	--	--	--
GluR3	--	--	--	--	--	--
Kainate 2 receptor	0.01 ↓	--	0.07 ↓	--	--	--
Hydroxysteroid 17-beta dehydrogenase 1	--	--	--	--	--	--
Hydroxysteroid 17-beta dehydrogenase 2	--	--	--	--	--	--
Hydroxysteroid 17-beta dehydrogenase 3	--	--	--	--	--	--
Hydroxysteroid 17-beta dehydrogenase 8	--	--	--	--	--	--
Insulin-like growth factor 1	--	**<0.001 ↑**	--	--	--	--
Insulin-like growth factor 1 receptor	0.03 ↓	--	0.04 ↓	--	--	--
Kisspeptin	--	--	--	--	--	--
Kisspeptin receptor (GPR54)	**<0.001 ↓**	0.01 ↓	**<0.001 ↓**	0.009 ↑	0.047 ↑	0.05 ↑
Membrane progesterone receptor	--	--	--	--	--	--
N-methyl-D-aspartate receptor (NMDAR) subunit 1 (NR1)	--	--	--	--	--	--
NMDAR subunit 2a	0.066 ↓	--	0.033 ↓	--	--	--

NMDAR subunit 2b	--	--	--	**<0.001 ↑**	**<0.001↑**	--
NMDAR subunit 2c	**<0.001↑**	--	--	--	--	0.03 ↑
NMDAR subunit 2d	--	--	--	--	--	--
Prodynorphin	--	--	--	0.008 ↓	--	0.01 ↓
Progesterone receptor	--	--	--	--	--	--
Vesicular glutamate transporter 1	--	--	0.006 ↓	--	--	--
Vesicular glutamate transporter 2	--	--	--	--	--	--
Steroid 5-alpha reductase 1	--	--	--	--	--	--
Signal transducer and activator of transcription 5B	--	--	--	--	--	--
Steroid sulfatase	--	--	--	--	--	--
Neurokinin B	--	--	--	--	--	--
Transforming growth factor alpha	--	--	**< 0.001 ↑**	--	--	--
Transforming growth factor beta-1	--	--	--	--	--	--
Uncoupling protein 2	--	--	--	--	--	--
Vitamin D receptor	--	--	--	--	0.005 ↑	0.016 ↑

Low-density PCR arrays were used to measure expression of 45 genes in whole POAs of P1 F1 female and male rats treated prenatally with EDCs. Statistical results are shown for significant effects and for trends in data. Because of the Bonferroni correction, the cut-off for a significant effect was set at $p < 0.001$. Not shown are results for internal controls 18S, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In addition, one gene was not detected in the assay: 3ß-hydroxysteroid dehydrogenase (HSD3b).

Figure 4.6: PCB Effects on P1 POA Gene Expression

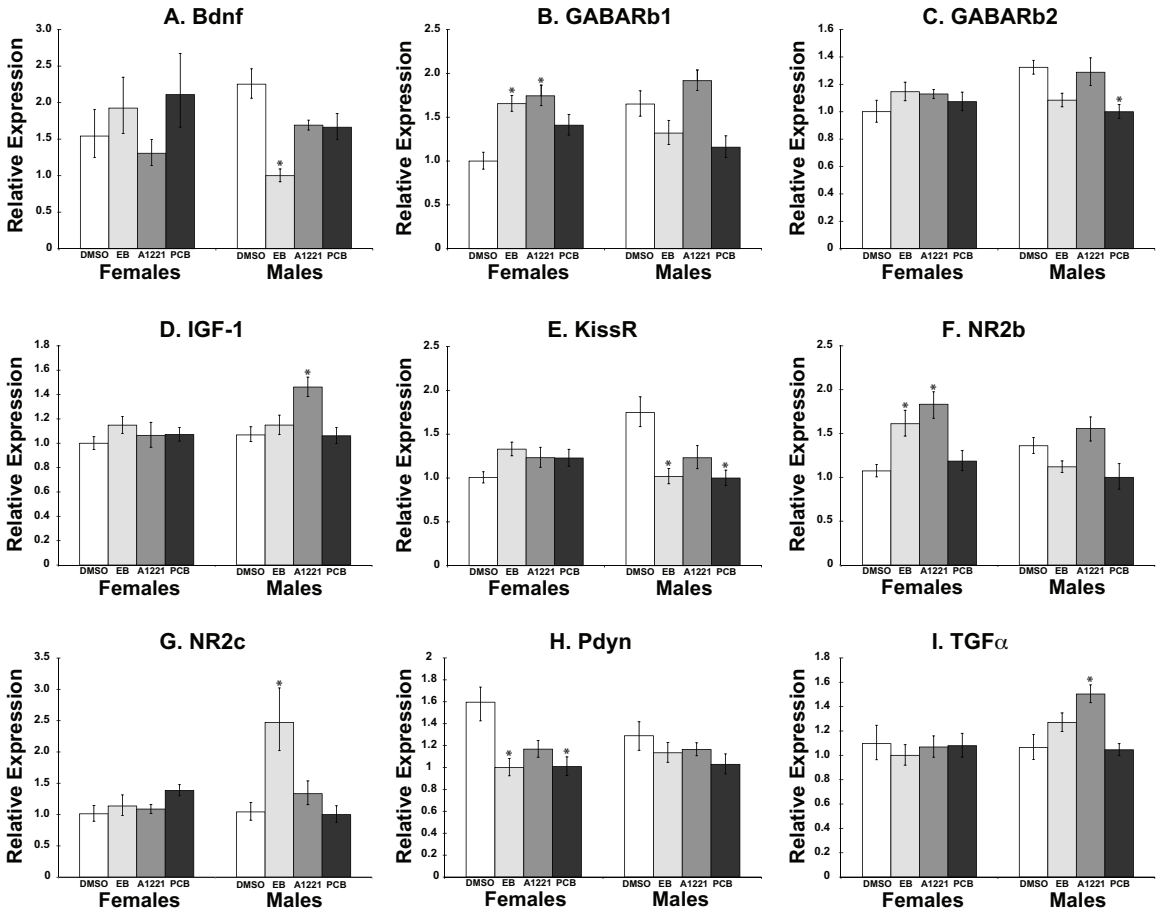

Figure 4.6: Gene expression data are shown for the nine neuroendocrine genes that were significantly affected in the POA of neonatal P1 male or female rats. Data shown are mean ± S.E.M. N = 6 rats per group; *p<0.001 vs. DMSO control of the same sex.

DISCUSSION

Neonatal exposure of developing organisms to EDCs such as PCBs is a global public health and environmental concern, as the body burdens of humans and wildlife, even in areas remote from industrialization, have appreciable levels of these compounds. PCBs readily cross the placenta and are passed to the suckling young in laboratory animals as well as humans (Masuda *et al*., 1978a; Masuda *et al*., 1978b; Ando *et al*., 1985; Lackmann *et al*., 1999; Covaci *et al*., 2002; Park *et al*., 2008). Moreover, due to their size and lipophilicity, PCBs cross the blood-brain-barrier, and accumulate in human fetal brain tissue at an average concentration of 50 ppb (Lanting *et al*., 1998). This is important, because exposure to environmental EDCs during critical developmental windows can cause permanent neuroendocrine deficits by disrupting hormonally regulated developmental processes, including brain sexual differentiation. We recently reported that prenatal PCB exposure impairs gene and protein expression in the adult female hypothalamus (Dickerson *et al*., 2011), and alters paced mating behavior (Steinberg *et al*., 2007). As sexual differentiation begins during this time period, our hypothesis is that PCB exposure at E16 and E18 causes immediate effects on developing sexually dimorphic regions of the neonatal brain. In support of our hypothesis, we found that low doses of PCBs, relevant for human exposures (Lackmann, 2002; Lackmann *et al*., 2004) had sex-dependent effects on developmental apoptosis in the P1 hypothalamus, as well as neuroendocrine gene and protein expression.

Effects on litter composition, AGD and body weight

In the current study, we selected doses of A1221 and PCB Mix to approximate human and environmental exposures (Lackmann, 2002; Lackmann *et al*., 2004), anticipating that these sub-toxic levels would not cause any gross morphological effects. Rather, our aim was to assess the early postnatal neuroendocrine outcomes of PCB treatment in the neonatal animal. This would enable us to establish just how early the brain is altered and the relationship between these early life outcomes to those we have reported for animals later in life (Steinberg *et al*., 2007; Steinberg *et al*., 2008; Dickerson *et al*., 2011). As predicted, EB, A1221, and PCB Mix had no significant effects on total or live births in the exposed F1 generation, nor did they affect sex ratio. In addition, we recorded body weights and measured the AGD of male and female pups on P1. Neonatal body weight is a reliable index of general pup health, as well as maternal nursing behavior and lactational capacity. A number of previous studies using PCBs have reported decreased birth weight in exposed laboratory animals (Turk and Hietman, 1976; Hany *et al*., 1999; Bowers *et al*., 2004; Shirota *et al*., 2006) and humans (Taylor *et al*., 1984; Murphy *et al*., 2010). However, we found no effect of treatment on birth weight with the current experimental parameters, although we did observe a trend toward increased body weight in females treated with either EB or A1221. The discrepancy in findings are likely due to experimental differences, including PCB dosage and congener composition, and timing, route, and duration of exposure. Littermates of the animals used for the current study were allowed to mature for a related study (Dickerson *et al*., 2011), and, similar to the trend we observed in neonatal females, we found a modest increase in

adult body weight in females prenatally exposed to either EB or A1221, but not in those exposed to the PCB Mix. Male body weight at adulthood was unaffected by treatment, as in the P1 animals described in this study.

In most mammalian species the AGD is sexually dimorphic, with males having a longer AGD than females. Neonatal anogenital distance is largely determined by the action of androgens (Marois, 1968), and thus can be an external indicator of masculinization and endocrine disruption (Gray *et al*., 1994). In the current study, we found a sex-dependent effect upon neonatal AGD, although not in the direction that we predicted: EB-, A1221-, and PCB Mix-treatment decreased (hyperfeminized) female AGD, while EB- and A1221-exposure increased (hypermasculinized) male AGD. We are not certain how to interpret this result. One possibility for the observed reduction in female AGD may be related to direct or indirect PCB effects on the androgen receptor (AR), although it is important to note that their relative binding affinity for the receptor is several orders of magnitude lower than endogenous androgens (Portigal *et al*., 2002; Fang *et al*., 2003). However, this cannot explain the larger AGD in EB-treated males. In the context of reduced serum testosterone observed in EB- and PCB Mix-treated males, the increase in male AGD may seem counterintuitive, but it is consistent with reports by our lab and others of increased AGD following developmental PCB exposure throughout postnatal development (Kuriyama and Chahoud, 2004; Dickerson *et al*., 2011).

Effects on serum hormones

Serum hormones were measured for the F1 generation. In females, none of the hormones measured were significantly affected by treatment with A1221 or PCB Mix, although EB treatment increased serum estradiol. However, it should be noted that variability in hormones was very high between the P1 pooled samples. In males, serum testosterone was reduced by EB and PCB Mix treatment at P1, while serum estradiol and progesterone were not affected by any treatment. Our results are consistent with previous studies that have reported a reduction in neonatal testosterone in male rats prenatally exposed to estrogenic chemicals or to PCBs. For instance, Takagi *et al*. (2004) found a reduction in testosterone at P3 in male rats exposed to ethinyl estradiol (Takagi *et al*., 2004), while Kaya *et al*. (2002) and Yamamoto *et al*. (2005) reported decreased serum testosterone in weanling male rats exposed to PCBs throughout the period of gestation and lactation (Kaya *et al*., 2002; Yamamoto *et al*., 2005a). Nevertheless, although the postnatal testosterone surge on the day of birth (Weisz and Ward, 1980) is critical for masculinization of the rodent brain, we observed male-typical neuroendocrine gene and protein expression in adult male littermates of these animals in a related study. It is possible that because we assayed testosterone on P1, the day following birth, we had poor temporal resolution for the testosterone surge that peaks the day of birth (Baum *et al*., 1988). Alternatively, it is possible that the reduction in testosterone observed in EB- or PCB-treated males reflected an increase in the metabolic breakdown of or sex steroid

hormone binding of free testosterone. Collectively, the results from the present and our related studies indicate that although postnatal testosterone may be transiently reduced by PCB treatment, exposed males nonetheless develop an appropriately masculinized AVPV during adulthood.

Effects on developmental apoptosis and ERα protein expression in the neonatal POA

In the current study, we investigated the effects of gestational PCB exposure on sexual differentiation and developmental apoptosis in the neonatal hypothalamus of males and females. In the female AVPV, treatment with EB or A1221, but not the PCB mix, increased the number of apoptotic nuclei, while no effect of treatment was observed in the male AVPV. In addition, no effect of treatment was detected in the MPN of either sex. The similar results for EB and A1221 suggest that these effects are exerted through an estrogenic mechanism to masculinize the female AVPV via increased apoptosis. In addition, the differences between A1221 and the PCB mix may be attributable to their difference in relative estrogenicity at the ER. While A1221 has been consistently shown to have estrogenic properties, the di-ortho substituted non-coplar congeners that comprise the reconstituted PCB mixture have demonstrated anti-estrogenic properties in several *in vitro* studies (Oh *et al*., 2007). The lack of treatment effect in males may be attributable to the higher baseline exposure of their developing hypothalamus to steroid hormones. The consequences of altering developmental apoptosis in the female AVPV may alter the AVPV's ability to generate the estrogen-induced GnRH/LH surge and ovulation in

adulthood (Wiegand *et al*., 1978; Wiegand and Terasawa, 1982; Gu and Simerly, 1997; Le *et al*., 2001). In our related study (Dickerson *et al*., 2011), we found that EB- and A1221-treatment reduced AVPV volume in female littermates of the animals used herein who had been allowed to mature to early adulthood and were studied at P60. Together, these results suggest that increased AVPV cell loss at P1 may be a contributing factor to the reduction of the regional AVPV volume observed in adult (P60) females.

There are also developmental sex differences in the number of cells in the AVPV that are immunopositive for ERα (Davis *et al*., 1996b; Orikasa and Sakuma, 2003). Thus, we also ascertained the effect of prenatal EDC exposure on this endpoint in the neonatal AVPV and MPN. In both regions, qualitative observations suggested no clear sex difference in ERα-ir at P1, consistent with reports from other labs (Yokosuka *et al*., 1997). Although we could not quantify ERα-ir in the AVPV due to low cell numbers, in the MPN, stereological analysis showed that treatment with EB, but not PCBs, increased the number of cells immunopositive for ERα in both male and female P1 pups. These results were unexpected, as estradiol has been shown to down-regulate ERα at the level of gene expression (Lauber *et al*., 1991; Simerly and Young, 1991) as well as immunoreactivity in the adult (Koch, 1990) and neonatal (P10) (Orikasa *et al*., 1994; Orikasa *et al*., 1995; Orikasa *et al*., 1996) rodent brain. Other studies investigating the effects of PCBs upon hypothalamic estrogen receptor expression are quite limited. In one, Lichtensteiger *et al*. (2003) found that gestational exposure to A1254 increased ERα gene expression in the ventromedial nucleus of the hypothalamus (VMN), a region important for feminine sexual behavior, in female rat embryos (Lichtensteiger *et al*., 2003). Because

sex differences in ERα in the AVPV and MPN regions do not appear until postnatal day 10, it is likely that future studies evaluating later developmental time points will provide temporal resolution to the endocrine disrupting effects of PCBs upon hypothalamic ERα. Although the experimental design did not enable us to collect tissues from rats at ages other than P1 (this study) and P60 (Dickerson *et al*., 2011), ongoing work includes a more systematic analysis of developmental postnatal profiling of the POAs of male and female rats prenatally exposed to PCBs.

EDCs affect neuroendocrine gene expression in the neonatal POA

Using a custom-designed 48-gene PCR array, we identified nine genes whose expression changed significantly with neonatal PCB exposure: brain-derived neurotrophic factor (BDNF), $GABA_B$ receptors 1 and 2, IGF-1, kisspeptin receptor, NMDA receptor subunits NR2b and NR2c, prodynorphin, and TGFα. Each of these identified genes is an important contributing factor to hypothalamic development, differentiation and function (Gore, 2001b; Daftary and Gore, 2004; Walker *et al*., 2009). For instance, BDNF stimulates migration of neurons during development, is highly expressed in discrete regions of the hypothalamus during postnatal development, and its release from certain cell types is regulated by gonadal steroid hormones [reviewed in (Tobet *et al*., 2009)]. In our study, expression of BDNF was reduced 50% by EB treatment in males, while females were not affected by treatment. Similarly, several neurotransmitters, including GABA, are thought to act as neurotrophic factors in

hypothalamus (McClellan *et al*., 2008). The $GABA_B$ receptor subunits B1 and B2 guide cell migration and positioning in the ventromedial nucleus (VMN). In females prenatally exposed to either EB or A1221, we observed an increase in the expression $GABA_{B1}$, while a decrease in the expression of $GABA_{B2}$ was observed in males treated with the PCB mix. This observation could have implications for proper establishment of sexually dimorphic circuitry and connections within this region.

Other significantly affected genes are involved in brain sexual differentiation by modulating cell survival and developmental apoptosis. For example, the growth hormone IGF-1 plays a crucial role in somatic growth, as well as proliferation and inhibition of apoptosis [reviewed in (D'Ercole and Ye, 2008)]. Similarly, NR2b and NR2c are each subunits of the ionotropic NMDA receptor, whose activation not only has actions on GnRH neurons and the reproductive axis (Gore, 2001b; Maffucci *et al*., 2008), but also play an important role in sexually dimorphic apoptosis (Hsu *et al*., 2000; Hsu *et al*., 2001).

Kisspeptin signaling is important for many aspects of reproductive maturation and function, including a recently discovered role in the guidance of GnRH neurites to the median eminence at the base of the hypothalamus (Fiorini and Jasoni, 2010). In males treated with either EB or PCB Mix, we observed a decrease in POA kisspeptin receptor expression. Interestingly, in littermates of these animals used for a related study, we observed a delay in male pubertal onset (Dickerson *et al*., 2011) that may reflect improper targeting of kisspeptin fibers. The neuropeptide dynorphin is co-expressed in kisspeptin neurons, and also acts to modulate GnRH secretion (Navarro *et al*., 2009a). In

females treated with EB or PCB Mix, we observed a decrease in prodynorphin gene expression, and a non-significant trend (p=0.006) in A1221-treated females. This observation is consistent with results of our related study, where we observed decreased kisspeptin protein expression and impaired GnRH neuron activation in adult female littermates in the EB, A1221, and PCB treatment groups (Dickerson *et al*., 2011). It is possible that disruption of this neuronal circuitry begins during neonatal development. Finally, TGFα, expression of which was increased in male-A1221 rats, is a cytokine involved in a number of cellular functions such as cell growth, proliferation, differentiation, and apoptosis (Galbiati *et al*., 2003). Moreover, this growth factor is released by hypothalamic glial cells, and plays a role in regulation of GnRH release. As a whole, these gene expression data identify a group of candidates for further study as early developmental targets of EDCs.

SUMMARY AND CONCLUSIONS

Collectively, these data show that endocrine disruption by gestational PCBs causes changes to the hypothalamic neural circuitry controlling reproduction, a process that is detectable as early as the day after birth. We found that PCBs cause changes in sexually dimorphic brain regions underlying sex-specific reproductive physiology and behavior through the perturbation of normal developmental apoptosis, and by altering gene expression of neurotransmitters and receptors known to play important roles in differentiation and migration of hypothalamic neurons. Although there may not be an

easy or obvious interpretation of the nine genes affected by prenatal PCB exposure, the gene expression data add to the story of the effects of developmental EDC exposure on reproductive neuroendocrine function. In fact, the sex differences in gene expression profiles and differential regulation by the various EDCs suggest that subtle mechanistic differences underlie the physiological phenotypic differences such as hormonal regulation, reproductive development, and adult reproductive function. Because proper neuronal migration and development requires that expression of downstream signal transduction components occur in temporal- and spatial-specific patterns, these changes in gene expression have important implications for sexual differentiation of the hypothalamus.

CHAPTER 5: DEVELOPMENTAL PCB EXPOSURE DISRUPTS SEXUAL DIFFERENTIATION OF THE RAT HYPOTHALAMUS

The text in this section is modified from the article "Endocrine disruption of brain sexual differentiation by developmental PCB exposure," Dickerson SM, Cunningham SL, Patisaul, HB, Woller, MJ, and Gore AC, (*Endocrinology*, 2011; in press).

ABSTRACT

In mammals, sexual differentiation of the hypothalamus occurs during prenatal and early postnatal development due in large part to sex differences in hormones. These early organizational processes are critically important for the attainment and maintenance of adult reproductive functions. We tested the hypothesis that perinatal exposure to polychlorinated biphenyls (PCBs) that disrupt hormonal pathways would perturb reproductive maturation and the sexually dimorphic development of neuroendocrine systems in the preoptic area (POA). Pregnant Sprague-Dawley rats were injected on gestational days 16 and 18 with vehicle (DMSO), Aroclor 1221 (A1221, an estrogenic PCB mix), a PCB mixture representing those highest in human body burden (PCBs 138, 153, 180) or estradiol benzoate (EB), an estrogenic control. Male and female pups were monitored for somatic and reproductive development. In adulthood, some rats were perfused and used for immunohistochemistry of estrogen receptor α (ERα), kisspeptin and co-expression of Fos in GnRH neurons. Other rats were used to obtain fresh-frozen POA dissections for use in a PCR-based 48-gene expression array. Pubertal onset was

advanced and estrous cyclicity irregular in endocrine-disrupted females. Furthermore, sexual differentiation of female neuroendocrine systems was masculinized/defeminized. Specifically, in the adult female anteroventral periventricular nucleus (AVPV), ERα cell numbers and kisspeptin fiber density were significantly decreased, as was GnRH-Fos co-expression. PCR analysis identified androgen receptor, IGF-1, NMDA receptor subunit NR2b, and TGFβ1 mRNAs as significantly down-regulated in endocrine-disrupted female POAs. These data suggest that developmental PCBs profoundly impair the sexual differentiation of the female hypothalamus.

INTRODUCTION

Exposure to environmental endocrine disrupting chemicals (EDCs) during susceptible periods of development, particularly embryogenesis and early postnatal life, has been shown across a wide range of species to cause neurological and reproductive deficits (reviewed in (Dickerson and Gore, 2007)). In humans, there is growing concern that exposure to low levels of EDCs may contribute to advanced pubertal onset in females and an overall decline in fertility in both sexes (Patisaul and Adewale, 2009). Polychlorinated biphenyls (PCBs) are a prototypical class of EDCs that are detectable in nearly all humans, and have been linked to a broad range of reproductive impairments. PCBs are of particular concern for developing offspring of exposed females, because a portion of the maternal body burden is transferred to the neonate through the placenta and mother's milk (Ando *et al*., 1985). The specific mechanisms underlying the observed

reproductive effects are not well understood, but many EDCs, including PCBs are thought to act through steroid hormone receptors, particularly the estrogen receptors (ERs) (Dickerson and Gore, 2007), to target neural and reproductive tissues.

In vertebrates, reproduction is coordinated by the hypothalamic-pituitary-gonadal (HPG) axis. At the neuroendocrine level, specific regions within the hypothalamus are sexually dimorphic, and function to coordinate sex-appropriate reproductive physiology and sexual behavior. In rodents, these sex differences in hypothalamic morphology and neurochemistry become organized during critical developmental windows, particularly the late embryonic and early postnatal periods, when sex differences in levels of gonadal hormones are large. While the female rodent brain develops in the relative absence of sex steroid hormones (Bakker and Brock, 2010), the brain of the neonatal mammalian male is exposed to higher levels of testosterone, along with the product of its aromatization, estradiol (McCarthy, 2008). Not only are cell numbers and brain regional volumes dimorphic, but the phenotype of cells is also sexually differentiated due to hormonal exposures. In rodents, one such region is the anteroventral periventricular nucleus (AVPV), which differs between males and females in regional volume, estrogen receptor α (ERα) and kisspeptin expression. This sexual dimorphism appears to underlie differences in positive feedback effects of estradiol, via AVPV ERα and kisspeptin-expressing neurons, on the hypothalamic GnRH neurons that control preovulatory LH release, which occurs in females but not in males. Furthermore, kisspeptin neurons located in the AVPV are important for pubertal initiation (Navarro *et al*., 2004; Kauffman

et al., 2007b) and such neurons are potential targets for the actions of EDCs (Bateman and Patisaul, 2008), although this latter question has not yet been addressed for PCBs.

In the current study, we evaluated the effects of perinatal exposure to low levels of PCBs on somatic and reproductive neuroendocrine development, including sexual differentiation of the AVPV. The PCB mixtures used in this study differ based on degree of chlorination, half-lives, and chemical properties. A1221 is a lightly chlorinated mixture of estrogenic PCBs that was once used commercially and has a half-life on the order of days (Matthews and Anderson, 1975). We also utilized a reconstituted mixture of more heavily chlorinated PCBs: PCB138, PCB153, and PCB180, which comprise the three most prevalent congeners detected in human and wildlife samples (Gladen *et al*., 2003). The congeners in this PCB mixture have been reported to be estrogenic, anti-estrogenic or anti-androgenic (Bonefeld-Jorgensen *et al*., 2001), and have a half-life on the order of years. At low doses, these compounds have been reported to shown to interact with steroid hormone receptors (Bonefeld-Jorgensen *et al*., 2001). Here, we performed immunohistochemical assessments of ERα, kisspeptin and GnRH, and we used a 48-gene PCR-based array (Walker *et al*., 2009) to identify novel hypothalamic gene expression targets of developmental PCB exposure, as manifested in the adult male and female rats.

MATERIALS AND METHODS

Animals and perinatal treatment

All experimental procedures were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals, and performed following protocols approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Three-month old Sprague-Dawley rats were purchased from Harlan Sprague–Dawley Inc. (Houston, Texas; Stock/Strain: Hsd:Sprague–Dawley®™ SD®™) and were housed individually in a humidity- and temperature-controlled room with a 12:12 partially reversed light cycled (lights on 2300 h, lights off 1100 h) at 21 – 23 °C. Because standard soy-based rat chows contain phytoestrogens, which may affect the neuroendocrine endpoints studied herein, animals were switched to low-phytoestrogen Harlan-Teklad 2019 Global Diet *ad libitum* for the duration of the experiments. Although this diet is not phytoestrogen-free, previous work from our lab has shown that rats of this strain and on this diet are sensitive to the endocrine disruptor treatments (Steinberg *et al*., 2007; Steinberg *et al*., 2008). Animals were handled daily to minimize stress and acclimatized to the new diet for at least three weeks before mating to generate F1 generations.

Female rats were mated with sexually experienced male rats (mated in a random rotation with females assigned to different treatments to avoid any paternal bias), and the day of sperm positive vaginal smears was termed embryonic day (E) 1. Dams (n=8 per treatment group) were randomly assigned to one of four treatment groups and injected

i.p. on E16 and E18 with one of four treatments (the same treatment given on E16 and E18): 0.1 ml of vehicle (DMSO 99.5%, Sigma, #D4540, Lot# 037K07663); 50 μg/kg estradiol benzoate (Sigma, #E8515, Lot# 125K1029, serving as an estrogenic positive control); 1 mg/kg Aroclor 1221 (AccuStandard, #C-221N, Lot# 083-166; dose based on our published work showing effects on reproductive function in female rats (Steinberg *et al*., 2007; Steinberg *et al*., 2008); or 1 mg/kg reconstituted PCB mixture (referred to as PCB Mix). The PCB Mix was composed of the three most prevalent PCB congeners found in mammalian tissue samples (Gladen *et al*., 2003; Bentzen *et al*., 2008): PCB138 (2,2',3,4,4',5'-Hexachlorobiphenyl; AccuStandard, #C138N, Lot# 082704MS-AC), PCB153 (2,2',4,4',5,5'-Hexachlorobiphenyl; AccuStandard, #C153N, Lot# 111804AG-AC), and PCB180 (2,2',3,4,4',5,5'- Heptachlorobiphenyl; AccuStandard, #C180N, Lot# 013004MT-AC), each present at the same concentration. These three congeners are nonplanar and do not bind the arylhydrocarbon receptor (AhR) with appreciable affinity (Van den Berg *et al*., 1998) The dose of PCB used was selected to approximate the typical body burden of newborn humans (Lanting *et al*., 1998; Lackmann, 2002). The timing of administration was selected to coincide with the period just prior to brain sexual differentiation during late gestation (Murakami and Arai, 1989; Rhees *et al*., 1990a). We chose intraperitoneal injection for continuity with previous reports, including our own, showing that this route of PCBs affects neuroendocrine endpoints (Chung and Clemens, 1999; Chung *et al*., 2001; Gore *et al*., 2002; Salama *et al*., 2003; Woodhouse and Cooke, 2004; Steinberg *et al*., 2008). Because individual pups are exposed to approximately 500X less than the maternal dose (Takagi *et al*., 1986; Steinberg *et al*., 2007; Steinberg *et*

al., 2008), we estimate that each pup was exposed to approximately 2 μg/kg total PCBs for both the A1221 treatment group and the PCB Mix treatment group. Although we did not quantify body burden of experimental animals following dosage of the pregnant dam, other laboratories have confirmed the transfer of injected PCB138, PCB153, and PCB180 from the dam to individual pups, as well as accumulation in the dam and pup brains (Cocchi *et al*., 2009). During pregnancy, dams were provided with nesting materials on E20, and were left undisturbed until the day after birth, termed postnatal day (P) 1, to minimize stress from handling that might interfere with parturition, maternal behavior, or add a confounding factor to the experiment.

Developmental measures

For each dam, weights during gestation and gestational duration were recorded, and these did not vary between treatment groups. All dams delivered spontaneously and the day of parturition, determined by the birth of at least one pup before lights out at 1100 h, was termed postnatal day (P) 0. On P1, the numbers of live and dead offspring, referred to as the F1 generation, were counted, sex ratio was determined, and the litter was standardized to 6 pups (three males and three females). Experimental animals for this study were selected based on anogenital distance (AGD); those three individuals with AGD closest to the litter median AGD per sex were retained, while the remaining animals were euthanized. AGD was measured using a digital caliper (Marois, 1968; Steinberg *et al*., 2008), and the ratio of AGD to the cube root of body weight (AGD index) was calculated to evaluate AGD (Marois, 1968; Gallavan *et al*., 1999). Tissues

from the culled P1 pups were banked for separate reproductive and neuroendocrine studies (not reported here).

After culling, the remaining F1 pups' AGD was measured on P7, P14, and P21. Body weight was measured on P7, P14, P21, P28, P35, P49, and P60. The day of eye opening was recorded. On P21, pups were weaned and housed 3 same-sex littermates per cage, and animals were checked daily for pubertal onset starting on P28. The timing of puberty was quantified as age of vaginal opening (VO), first estrus (FE), and first diestrus (FD) (Salama *et al*., 2003; Steinberg *et al*., 2008) for females, and age at preputial separation for males (Korenbrot *et al*., 1977). In females, daily vaginal lavage was conducted from the day of vaginal opening until euthanasia. Intact females were euthanized on the closest age following P58 at which proestrus occurred.

Tissue collection

At approximately P60, one male and female F1 rat per litter was euthanized for protein studies (N=8 per sex, each from different litters), and 1 male and female per litter was utilized for gene expression studies (N=6 per sex, each from different litters). For gene expression analyses, animals were rapidly euthanized by decapitation between 0900 – 1000 h (males), or on the first proestrus following P58 between 1500 – 1700 h (females). Brains were quickly removed and the preoptic area-anterior hypothalamus (POA), which contains the AVPV and GnRH cell bodies, was dissected on ice, snap frozen (Dickerson *et al*., 2008) and stored at -80 C until processing. Trunk blood was

collected, allowed to clot, centrifuged for 5 min at 5000 X g, and stored at -80 C until use.

For immunohistochemistry studies, F1 animals were deeply anesthetized with 0.4 ml ketamine (100 mg/ml) and 0.4 ml of xylazine (20 mg/ml), and trans-cardially perfused initially with 0.9% saline (50 ml) at a rate of 50 ml/min, followed by 4% paraformaldehyde (500 ml). Following perfusion, the brains were carefully removed and postfixed overnight in 4% paraformaldehyde, and then transferred into PBSA with 0.2% sodium azide for storage at 4 °C. A vibrating microtome (Leica VT 1000S, Leica Microsystems, Nussloch, Germany) was used to cut brain into sections (40 μm-thick) that were stored in PBS with 0.2% sodium azide at 4 °C until use within a 6-month period. At the time of perfusion, a cardiac puncture was performed on the anesthetized rats to collect a terminal blood sample, and after perfusion the uteri, ovaries, and testes were removed and wet weights were recorded. We note that during perfusion the descending aorta is clamped such that while the head and brain are fixed, the trunk (including organs) are unfixed to enable wet weight measurements and tissue collection. One ovary or one testis from each animal was post-fixed in Bouin's solution for morphological analysis. Blood samples were centrifuged at 5000 x g for five minutes to separate serum, and serum was stored at -80 C until steroid hormone analysis. Males were euthanized between 0900 – 1000 h, and females were euthanized on the first proestrus following P58 between 1500 – 1700 h. For either method of euthanasia, all carcasses were examined for any gross abnormalities, and none were found.

Gonadal morphology

Ovaries and testes of F1 animals were coded at collection, and processed at the NIEHS Center for Research on Environmental Disease (CRED) Histology and Tissue Processing Facility Core. For each animal, the ovary or testis was paraffin embedded, microtome sectioned at 6 μm, and mounted onto slides (Superfrost Plus, Fisher Scientific). Sections were then deparaffinized, stained with hematoxylin and eosin and coverslipped. For males, random testis sections (2 per male) were qualitatively examined for histological abnormalities, and for presence of mature sperm. For females, ovary sections (2 per female) were qualitatively examined for histological abnormalities, and for the number of corpora lutea.

Serum hormone assays

LH concentrations in serum samples of F1 adults was measured in the laboratory of Dr. Michael Woller, University of Wisconsin-Whitewater, by double antibody competitive binding RIA as previously described (Vella *et al*., 2001; Maffucci *et al*., 2008), using reagents provided by Dr. A. F. Parlow of the National Hormone and Pituitary Program at NIDDK. The reference standard used was rat LH-RP-3. Duplicate volumes of 100 μl serum were used for each sample, and a single assay was performed. The assay sensitivity was 0.2 ng/ml, and the intra-assay coefficient of variability (CV) based on duplicate samples was 4.33%.

Total serum testosterone was determined in a single assay using the Active® Testosterone coated well EIA kit (Catalog # DSL-10-4000, Lot # 08035-B, Diagnostic

Systems Laboratories, Inc., Webster, TX, USA), according to the manufacturer's instructions. Duplicate volumes of 50 μl serum were used for each sample. The assay limit of detection was 0.04 ng/ml, and the intra-assay CV based on duplicate samples for the assay was 2.97%.

Progesterone concentrations were determined in a single assay using the ACTIVE® Progesterone Coated-Tube Radioimmunoassay Kit (Catalog # DSL-3900, Lot # 07076, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), according to the manufacturer's instructions. Duplicate volumes of 25 μl serum were used for each sample. The sensitivity of the assay was 0.12 ng/ml, and the intra-assay CV was 3.26%.

Estradiol concentrations were determined in a single assay using an ultrasensitive double-antibody RIA kit (Catalog # DSL-4800, Lot # 07076, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), according to the manufacturer's instructions. Duplicate volumes of 200μL serum were used for each sample. The assay limit of detection was 2.2 pg/mL, and the intra-assay coefficient of variability based on duplicate samples was 7.97%. For all assays, a few samples for which the CV between duplicates was 10% or greater were excluded from analysis.

Immunohistochemistry of ERα, kisspeptin, and GnRH-Fos double labeling

All analyses were performed on coded sections by investigators blind to treatment. For ERα immunohistochemistry, sections were selected in a 1:2 series through the AVPV of adult male and female F1 rats, and were processed in four runs using the purified rabbit polyclonal antibody C1355 (1:20,000, Upstate Biotechnology, Waltham,

MA) as previously described in detail (Chakraborty *et al*., 2003; Wu *et al*., 2009a). The antibody is well characterized and has no cross-reactivity with ERβ (Wu *et al*., 2009a). For each run, animals from each sex and treatment group were equally represented. In order to make the experimenter blind to sex and treatment group, section IDs were recoded. All steps, unless noted, occurred at room temperature (22 °C) on a shaking platform. Procedures of immunohistochemistry, tissue handling and Nissl staining were done identically to our previously published work (Wu *et al*., 2009a). In brief, sections were incubated in the primary antibody for 48 hours, incubated with 5% normal goat serum and secondary antibody (biotinylated goat anti-rabbit immunoglobulin (Ig)G, 1:600; BA-1000; Vector Laboratories) for one hour, then subjected to peroxidase reaction with nickel-enhanced DAB. Sections were mounted on gelatin-subbed slide, dried, dehydrated in a graded alcohol series, counterstained with methyl green, and coverslipped with DPX (44581; Fluka, Steinheim, Germany).

For each adult F1 animal, three AVPV tissues per rat were immunolabeled for kisspeptin using immunofluorescence methods described in detail elsewhere (Bateman and Patisaul, 2008). In brief, tissues were incubated with the rabbit polyclonal kisspeptin-10 antibody (1:6000; generous gift of Dr. Alain Caraty, Institut National de la Recherche Agronomique/Centre National de la Recherche Scientifique, Université de Tours) for 48 hours. The antibody is well characterized for use in neural tissue and has been extensively validated for specificity by other labs (Gottsch *et al*., 2004; Smith *et al*., 2005; Clarkson and Herbison, 2006; Franceschini *et al*., 2006). The secondary antibody was the Alexa-Fluor goat anti-rabbit 488 (1:200, 2 hours). Tissues were mounted onto

slides (Superfrost Plus, Fisher, Pittsburgh, PA), and cover-slipped using VectaShield (Vector Labs).

Female adult F1 rats were perfused 3 hours after lights out on proestrus, when GnRH-Fos double-labeling is high (Finn *et al*., 1998). GnRH and Fos coexpression were detected using a cocktail of primary antibodies directed against GnRH (raised in mouse, 1:1,000, Santa Cruz, SC-32292) and Fos (raised in rabbit, 1:250, Santa Cruz, SC-52) followed by the secondary antibodies biotinylated horse-anti-mouse and biotinlyated goat-anti-rabbit, each at 1:600. After secondary antibody incubation, sections were processed with the Universal Elite ABC kit (Vector Laboratories, Burlingame, CA) and labeled with diaminobenzidine (brown) for GnRH and nickel-enhanced diaminobenzidine (black) for Fos. Sections were then rinsed, mounted onto slides (Superfrost Plus, Fisher, Pittsburgh, PA), and coverslipped using DPX mountant. Preliminary IHC showed that GnRH and Fos immunoreactivity was evenly distributed laterally and dorsally throughout the rostral to caudal extent of the organum vasculosum of the lamina terminalis (OVLT) (Wray and Hoffman, 1986; Adewale *et al*., 2009). Therefore, for each female two midlevel sections containing the OVLT through the caudal border of the AVPV were selected for immunolabeling and analysis of GnRH and Fos. Cells immunostained for GnRH only, and cells immunolabeled for both GnRH and Fos were counted by an individual blind to the treatment groups and verified by a second independent observer. GnRH neuron activation is expressed as percentage dual-labeled GnRH neurons out of the total number GnRH neurons counted.

Quantification of ER alpha cell numbers, kisspeptin fiber density, and GnRH-Fos double labeling

Quantification of ERα in the AVPV was performed using unbiased stereological analysis according to methods described in detail previously (Chakraborty *et al*., 2003; Chakraborty *et al*., 2005). A wet-mount of fresh tissue showed that average tissue thickness was 39.8 μm. The sections were carefully matched for rostral–caudal landmarks among all the animals, and the regions were identified in Nissl-stained sections by comparing anatomical landmarks to an atlas of the rat brain (Swanson, 1998). Contours were drawn around the AVPV at low magnification (10× objective) using an Olympus BX-61 microscope. A buffer zone at the top and bottom of sections was set at 3 μm for all experimental stereology. For each rat, the regional volume of the AVPV was extrapolated based on the contours and tissue thickness (Volume = regional area × thickness). The Stereo Investigator® software (MicroBrightField, Williston, VT) randomly placed 60 μm × 125 μm grids ("disector frames") within the AVPV contour. Within these disector frames, the DAB-stained ERα-labeled nuclei were counted within a 45 μm × 45 μm counting frame ("optical disectors"). Based on these parameters, the number and density (# immunoreactive cells/volume of each nucleus) of ERα-immunoreactive (ERα-ir) nuclei falling within the regions was quantified. The coefficients of error (Cruz-Orive/Geiser) and variation of the estimates were calculated as described by Schmitz and Hof (Schmitz and Hof, 2000). Photomicrographs were taken to produce the figures, and images were subjected to only minor adjustments of contrast

using Adobe Photoshop CS4 (Adobe, San Jose, CA). In order to avoid any bias, any adjustments were applied equally to tissues from rats of different treatment groups.

For quantification of kisspeptin fiber density, three AVPV sections were analyzed for kisspeptin immunofluorescence on a Leica TCS SPE confocal microscope fitted with a 63× oil corrective objective lens. For each scan, a set of serial image planes (z-step distance = 1 μm) was collected through the entire thickness of each section, producing a set of confocal stacks ranging in size from 26 to 36 image planes. Image stacks were analyzed using the Image J software package (National Institutes of Health (NIH), Bethesda, MD) as previously described (Bateman and Patisaul, 2008). To control for variations in tissue thickness that would result in unequal numbers of image planes, substacks of consecutive image planes that excluded the rostral and caudal edges of the tissue sections were created for each set of scans. Substacks consisted of 24 sequential image planes for the AVPV. Only data from sections with consistent staining throughout the entire thickness were included in the analysis. Individual images contained within each substack were first binarized and depixelated to minimize the inclusion of background fluorescence. Fibers were then skeletonized to a thickness of one pixel to compensate for differences in individual fiber thickness and brightness. The number of resulting bright pixels in each image plane was then quantified using the Image J Voxel Counter plug-in (NIH). The voxel counts were averaged within the substack to obtain a single measure for each section that was then used as a quantitative representation of average kisspeptin fiber density within the volume sampled (Bateman and Patisaul, 2008).

For quantification of GnRH neuron activation, GnRH/Fos double-labeled slides were visualized and evaluated with an Olympus BX-61 microscope. Each GnRH cell was scored as Fos-positive or negative based on the presence or absence of nickel-DAB product in the GnRH nucleus.

RNA extraction and Taqman low-density arrays (TLDA)

In brief, RNA from frozen POA dissections was extracted using an in-house double detergent lysis buffer system as described previously (Dickerson *et al*., 2008; Walker *et al*., 2009). Samples were homogenized and the cytoplasmic RNA was treated with proteinase K, followed by extraction with phenol chloroform and precipitation in isopropanol. Genomic DNA contamination was removed using the TURBO DNA-free kit (Applied Biosystems Inc, Foster City, CA) according to the manufacturer's protocol. The concentration of resulting cytoplasmic RNA was determined using a Nanodrop (ND-1000, Nanodrop Technologies, Inc., Wilmington, DE).

Samples were run on a custom-designed rat neuroendocrine TLDA (Applied Biosystems Inc., Foster City, CA), a panel of 48 candidate neuroendocrine genes chosen by our laboratory to identify putative genes in the POA affected by developmental EDC exposure. Cytoplasmic RNA (2 μg) was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA was stored at -20 C until use, at which time samples were diluted 1:5 before PCR reactions were performed using Applied Biosystems' Taqman reagents, and run on an ABI 7900 real-time PCR machine using the following parameters: 50 C for 2 min, 94.5 C for 10 min, 45

cycles of 97 C for 30 sec, and 59.7 C for 1 min. Relative expression for each gene was determined using the comparative Ct method (Pfaffl, 2001). Each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, a "control" gene chosen because it is unaffected by sex/treatment in the POA in this and another study (Walker *et al*., 2009), and the data were calibrated to the average change in Ct for the treatment group with the lowest expression.

Statistical analysis

SPSS statistical software (17.0 for Macintosh, SPSS Inc., Chicago, IL) was used to evaluate the effects of treatment on somatic and reproductive development, serum hormones, AVPV volume, AVPV ERα immunoreactivity and kisspeptin fiber density. Repeated-measures ANOVA was used to assess significant differences between treatment groups for serially recorded anogenital distances and body weights. Because previous research has already demonstrated sex differences in many of the neuroendocrine endpoints evaluated herein and we had independent *a priori* hypotheses for each sex, statistics were performed separately for each sex. Each dataset was tested for homogeneity of variance and normality. For datasets that met these criteria, comparisons were made by one-way ANOVA (factor: treatment) followed by Fisher's LSD post-hoc analysis when indicated by a significant main effect. Datasets for which variance between treatment groups was unequal were compared using the nonparametric Kruskal-Wallis test. For analysis of estrous cyclicity, statistical significance was determined using the nonparametric Fisher's exact test (Wu and Gore, 2010). For GnRH

neuron activation in females, percentage of GnRH and Fos co-labeled cells were compared across treatment groups by χ^2 analysis. The number of GnRH neurons counted did not differ between treatment groups (data not shown). In all these cases *p*-values < 0.05 were considered statistically significant. For the gene expression data, statistics were conducted using the normalized Ct (δ-Ct) for each sample (before transformation to fold change). Because the TLDA measures expression of 48 genes, a Bonferroni correction was used to adjust the alpha for significance to $p < 0.001$. The Grubb's test was used to detect a few outliers, which were excluded from statistical analysis.

RESULTS

Somatic and reproductive development

After animals were culled on P1, anogenital distance (AGD) for the remaining experimental animals was measured from the day after birth until weaning, and body weight was measured throughout postnatal development until P60. As shown in **Figure 5.1**, a slight but significant main effect of treatment ($p<0.01$) on postnatal body weight gain was detected in females, but not males, throughout postnatal development. Post-hoc analysis showed that females treated with EB ($p = 0.001$) or A1221 ($p=0.01$) were heavier than control animals. Moreover, neonatal EDC exposure resulted in a significant main effect of treatment ($p<0.01$) on male, but not female, AGD ($p<0.001$). Post-hoc analysis revealed an increase in AGD in EB-, A1221-, and PCB Mix-treated males throughout postnatal development ($p<0.01$; **Figure 5.1**).

As shown in **Table 5**, a significant main effect of treatment was observed on the developmental landmark of eye opening ($p < 0.05$) in females. Post-hoc analysis showed a modest but significant advancement in eye opening in females from the A1221 ($p<0.001$) and PCB Mix ($p<0.05$) groups compared to controls. We observed a significant interaction of treatment on pubertal onset ($p < 0.001$), with females prenatally exposed to EB ($p<0.005$), A1221 ($p<0.005$), or PCB Mix ($p<0.01$) having earlier vaginal opening compared to control females. Males treated with these same agents had a delay in preputial separation compared to control males (**Table 5**; $p<0.005$). The age of vaginal opening occurred an average of 1.4 days earlier in EDC-treated females than the control rats, while the age of preputial separation was 1.7 days later in EDC-treated males compared to control rats (**Table 5**).

Figure 5.1: Postnatal Anogenital Distance and Body Weight Gain

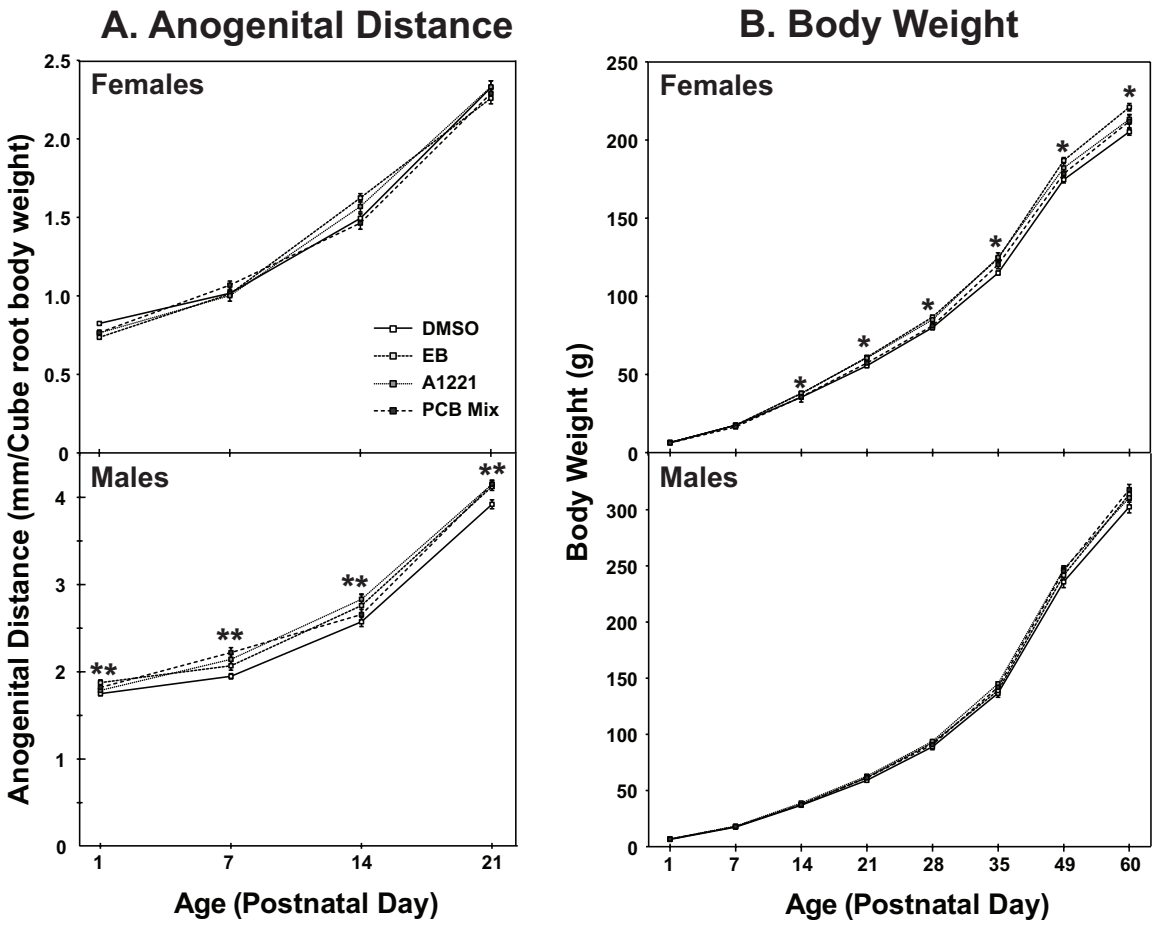

Figure 5.1: Postnatal Developmental landmarks. Data for anogenital distance (A) and body weight (B) shown are in postnatal days, mean ± S.E.M. Repeated-measures ANOVA showed that treatment with estradiol benzoate (EB), Aroclor 1221 (A1221), or the reconstituted PCB mixture (PCB Mix) caused a modest but significant increase in male AGD (**$p<0.001$), while female AGD was unaffected by treatment. For body weight, a slight but significant increase in body weight in EB- and A1221-treated females was detected. AGD index is the ratio of AGD (mm) to the cube root of body weight. The legend in the upper left panel applies to the other three panels.

Table 5. Summary of EDC Effects on Reproductive and Somatic Developmental Endpoints

A. Females				
	DMSO Vehicle	**Estradiol Benzoate**	**Aroclor 1221**	**PCB Mix**
Eye Opening (days)	15.07 ± 0.13	15.00 ± 0.15	14.36 ± 0.14 **	14.69 ± 0.14
Vaginal Opening (days)	34.14 ± 0.39	32.71 ± 0.30 **	32.64 ± 0.28 **	32.77 ± 0.38 *
First Estrus (days)	34.21 ± 0.41	33.29 ± 0.33	33.07 ± 0.30	34.54 ± 0.71
First Diestrus (days)	35.14 ± 0.37	34.29 ± 0.28	33.93 ± 0.41	35.00 ± 0.63
% Females with Irregular Estrous Cycles	0%	46% *	33% *	44% *
Uterus Wet Weight (g)	0.77 ± 0.06	0.72 ± 0.05	0.66 ± 0.07	0.78 ± 0.07
Ovary Wet Weight (g)	0.127 ± 0.004	0.129 ± 0.004	0.123 ± 0.003	0.147 ± 0.005 **
Gonadosomatic Index	0.61 ± 0.02	0.57 ± 0.02	0.57 ± 0.01	0.69 ± 0.03 *
B. Males				
	DMSO Vehicle	**Estradiol Benzoate**	**Aroclor 1221**	**PCB Mix**
Eye Opening (days)	15.07 ± 0.17	15.07 ± 0.16	14.79 ± 0.12	14.83 ± 0.12
Preputial Separation (days)	40.93 ± 0.23	42.67 ± 0.33 **	42.79 ± 0.41 **	42.33 ± 0.45 *
Paired Testes Wet Weight (g)	3.77 ± 0.004	3.68 ± 0.004	3.58 ± 0.003	3.68 ± 0.005
Gonadosomatic Index	1.23 ± 0.02	1.18 ± 0.02	1.15 ± 0.01	1.16 ± 0.03

Data shown are mean ± SEM. *p < 0.05, **p < 0.005 vs. DMSO vehicle.

Gonadosomatic index = gonad wet weight/body weight

Estrous cyclicity in females

Upon vaginal opening, regularity of the estrous cycle was assessed by daily vaginal lavage. An abnormal estrous cycle was defined as 3 or more consecutive days in estrus, or 4 or more consecutive days of diestrus in a cycle (Delclos *et al*., 2009). The nonparametric Fisher's exact test showed a treatment effect of EDCs on estrous cyclicity, with all groups of EDC-treated females having a significantly higher percentage of individuals having at least one irregular estrous cycle (**Table 5**; $\chi^2 = 17.1$, df = 3, $p<0.001$).

Gonadal weight and morphology

Gonadal weights and gonadosomatic index (calculated as [gonadal weight/body weight] * 100) were also affected in a sex-dependent manner by EDCs (**Table 5**). Ovarian weight ($p<0.01$) and gonadosomatic index ($p<0.05$) showed a slight but significant increase in PCB Mix-treated females, but gonadal weight was unaffected by treatment in males. There was a nonsignificant trend for male gonadosomatic index to be decreased by either PCB treatment ($p=0.051$). Gonadal morphology was not significantly different between treatment groups in adult males or females (**Figure 5.2**). Qualitative histological comparison between groups showed consistently healthy-appearing ovaries characterized by all stages of follicular development and the presence of healthy corpora lutea (CLs). None of the treatment groups displayed any degree of abnormalities such as hemorrhagic follicles, large antral-like follicles, multioocyte follicles, or ovarian cysts. In

females, the number of corpora lutea (CL) were counted to determine whether females had ovulated. Although there was a non-significant trend for a reduction in the number of CLs present in the A1221 and PCB Mix groups, this did not reach significance (DMSO= 13.75 ± 0.86, EB= 12.5 ± 2.3, A1221= 11.25 ± 3.1, PCB= 10.5 ± 1.7). In males, qualitative observations of testes showed no histological abnormalities in any of the treatment groups, nor any apparent differences in the presence of mature sperm (**Fig. 5.2**).

Effects of PCBs on serum hormones

Serum LH levels varied considerably among rats, an observation likely due to the pulsatile nature of LH release (Maffucci *et al*., 2008). Kruskal-Wallis analysis showed no effect of treatment upon LH levels in males or females (**Fig. 5.3A**). For estradiol, no main effects of treatment were observed in males or females (**Fig. 5.3B**). A significant main effect treatment on serum testosterone was detected ($p<0.05$) attributable to males treated with PCB Mix having lower levels of testosterone than A1221-treated male rats ($p=0.013$; **Fig. 5.3C**). However, no EDC groups differed from the DMSO control group. Serum progesterone levels were significantly reduced in males treated with either A1221 ($p=0.01$) or PCB-treated ($p=0.002$) animals (**Fig. 5.3D**).

Figure 5.2: EDC Effect on Gonadal Morphology

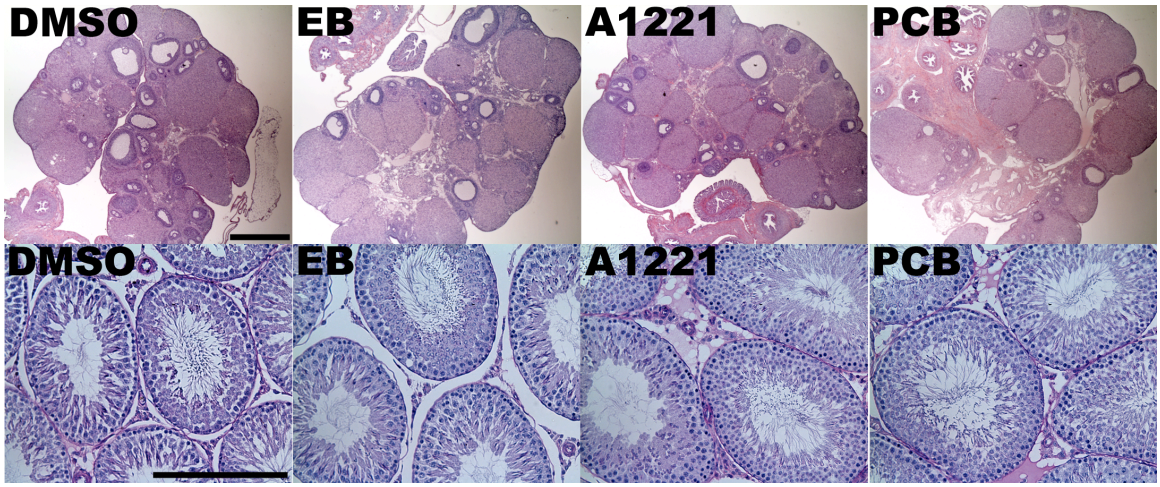

Figure 5.2: Representative photomicrographs of gonadal morphology from females (top row; scale bar = 500 μm) and males (bottom row; scale bar = 250 μm). Ovarian morphology did not differ significantly across treatment groups, and markers of active oogenesis and ovulation were observed in all samples. Testicular morphology was similar across treatment groups, with mature sperm observed in all samples.

Figure 5.3: EDC Effect on Adult Serum Hormones

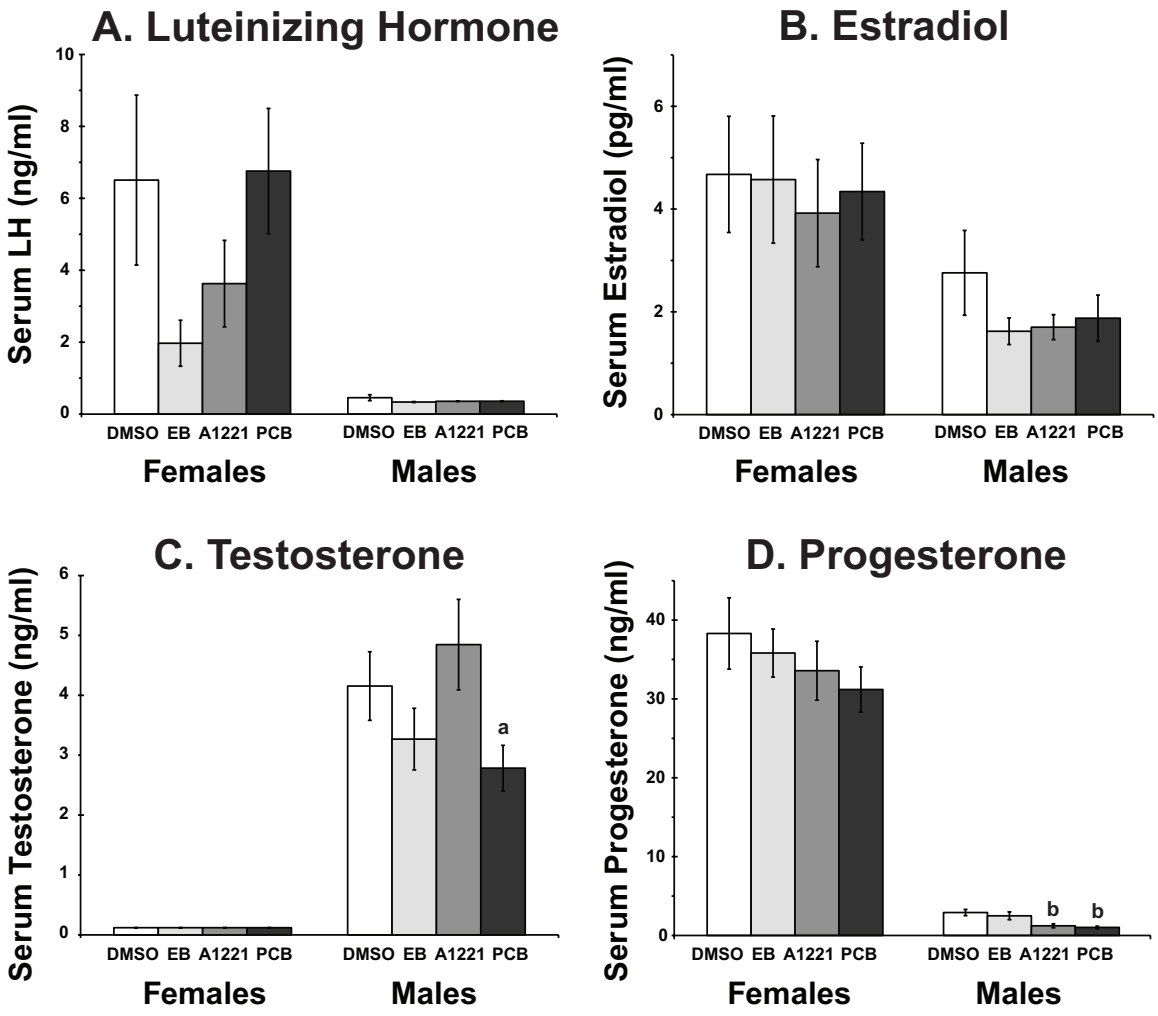

Figure 5.3: Serum hormone levels of luteinizing hormone (A), estradiol (B), testosterone (C), and progesterone (D) in adult F1 animals. For both LH and estradiol, no significant main effect of treatment upon either hormone was observed in males or females. For testosterone, A1221-treated males had higher serum testosterone compared to PCB Mix-treated males ($p<0.05$). For progesterone, A1221- and PCB Mix-treated males had lower levels than DMSO males. The bars represent the mean ± S.E.M. a, $p<0.05$ vs. A1221 males; b, $p<0.05$ vs. DMSO males. PCB on the x-axis refers to the PCB Mix group.

ER alpha and kisspeptin expression in the AVPV

Representative photomicrographs of ERα immunoreactivity in the AVPV are shown in male and female rats for the four treatments. (**Fig. 5.4, A-H**). Stereological cell counting showed a significant main effect of treatment on ERα-ir cell numbers in females, with EB- and A1221-treated females having significantly fewer cells than DMSO- and PCB Mix-treated females ($p<0.01$; **Fig. 5.5**). In addition, we observed a significant main effect of treatment upon AVPV volume in both males ($p=0.019$) and females ($p=0.01$). In females, treatment with EB ($p<0.01$), A1221 ($p<0.01$), or PCB Mix ($p=0.049$) resulted in reduced volume compared to DMSO females. PCB Mix-treated males had increased AVPV volume compared to DMSO males ($p<0.005$). Density of ERα was unaffected by treatment in AVPV of F1 males or females (**Fig. 5.5**).

Representative photomicrographs of kisspeptin immunoreactivity in the AVPV are shown in male and female rats (**Fig. 5.4**, **I-P**). We detected a significant effect of treatment on kisspeptin-ir density in females, but not males, with all EDC-treated F1 females having lower kisspeptin expression compared to control counterparts (**Fig. 5.6**; $p<0.001$).

Figure 5.4:Representative AVPV ERα and Kisspeptin Labeling

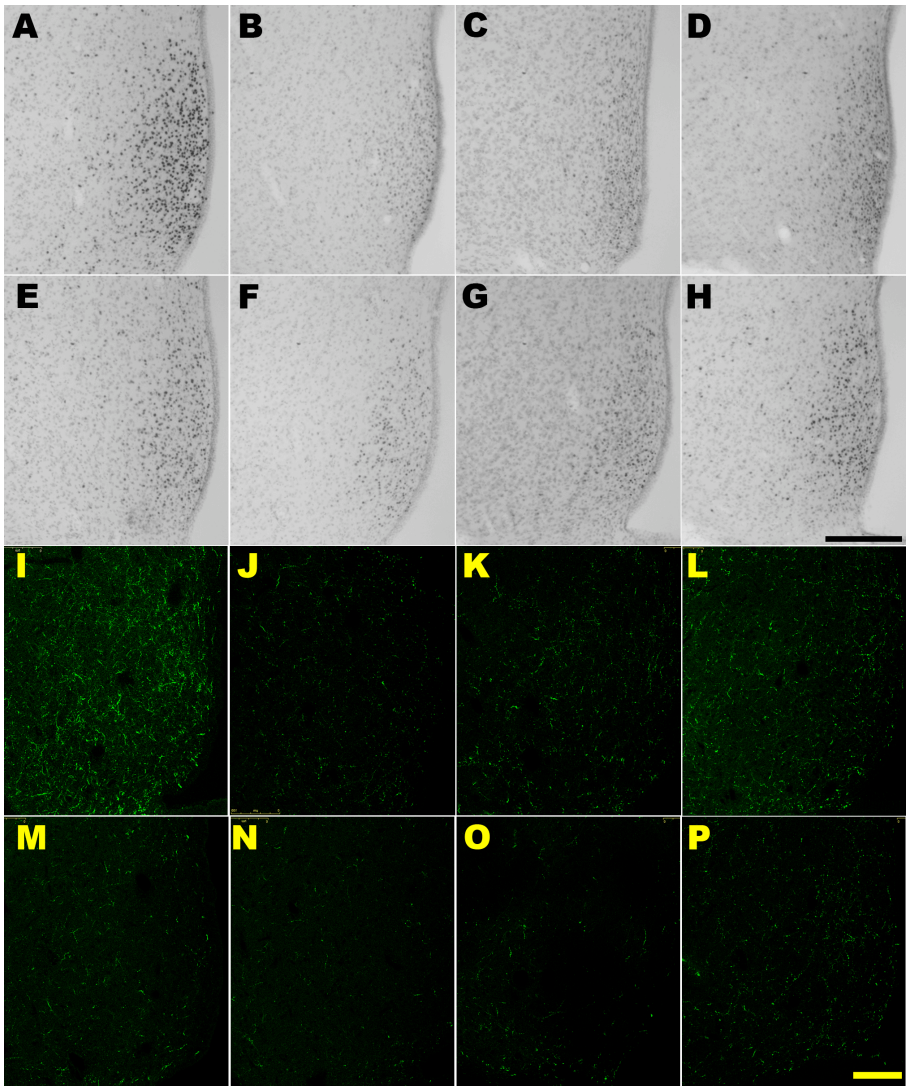

Figure 5.4: Upper Panel. Photomicrographs of estrogen receptor alpha (ERα) immunoreactivity in the anteroventral periventricular nucleus (AVPV) of P60 female and male rats developmentally exposed to DMSO vehicle (A, E), estradiol benzoate (B, F), A1221 (C, G), or PCB Mix (D, H). The third ventricle can be seen at the right of each micrograph. Although the images presented here were photographed at low magnification (10X), quantification and analysis of ERα-immunoreactivity was performed at high-power magnification (40X). ERα nuclei are labeled with dark brown nickel-enhanced diaminobenzidine product. Scale bar = 250μm. Lower Panel. Confocal images (5 merged consecutive optical planes) of kisspeptin fiber immunofluorescence in the AVPV of representative female and male rats. The third ventricle is at the right of each micrograph. Data are shown for a representative female and male treated prenatally with DMSO vehicle (I, M), EB (J, N), A1221 (K, O) and PCB Mix (L, P). Scale bar = 100μm.

Figure 5.5: PCB Effect on AVPV ERα Expression

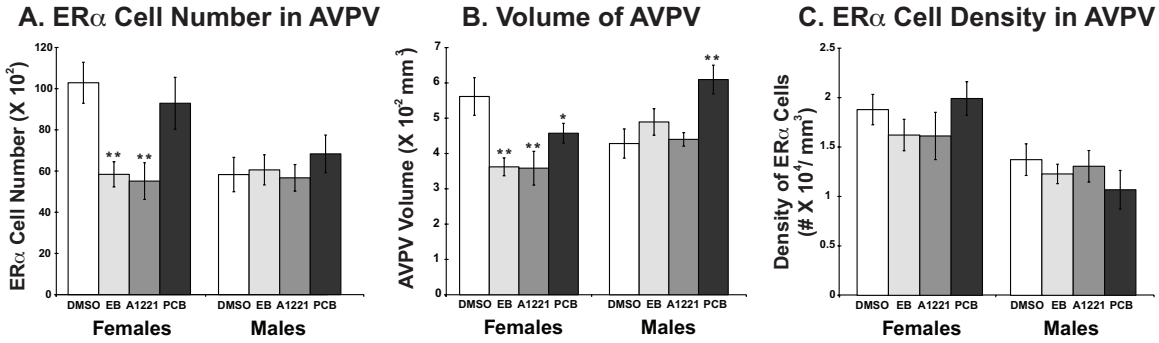

Figure 5.5: Stereologic analysis results for ERα-immunoreactive cell numbers in the AVPV (A), regional volume of the AVPV (B) and cell density (C) are shown for adult F1 rats in the AVPV (n = 8 to 10 rats per treatment group). Data are the mean ± S.E.M. ERα cell number was significantly reduced in EB- and A1221-treated females compared to DMSO control. Female AVPV volume was reduced by EDC treatment, while the AVPV volume of PCB Mix-treated males was higher than their control counterparts. Cell density was unaffected by treatment in either sex. *, $p < 0.05$ vs. DMSO control; **, $p < 0.005$ vs. DMSO control. PCB on the x-axis refers to the PCB Mix group.

Figure 5.6: EDC Effect on AVPV Kisspeptin Expression

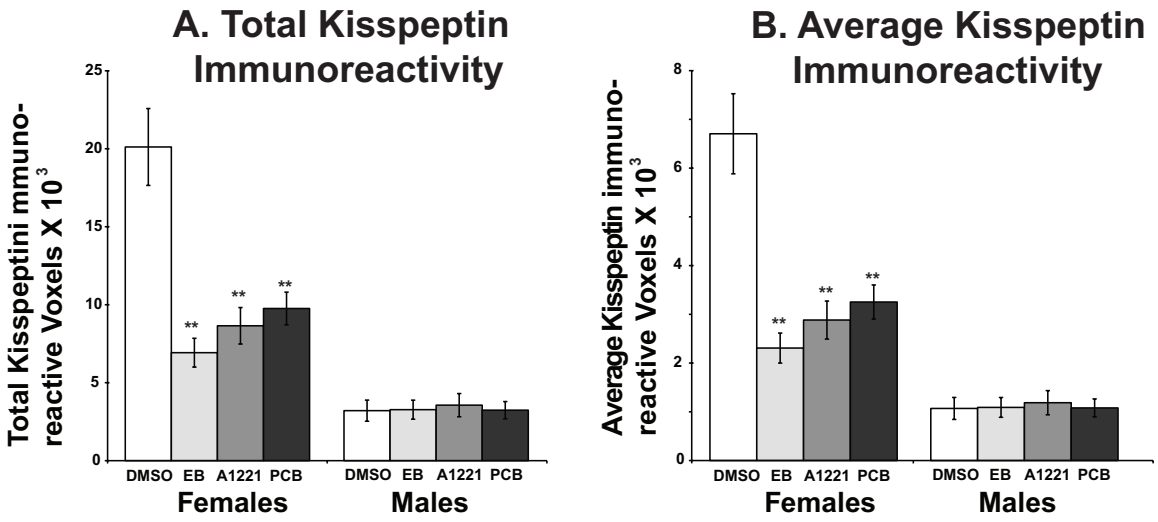

Figure 5.6: Quantification of kisspeptin immunofluorescence density in the AVPV. Data are shown for total (A) and average (B) kisspeptin immunoreactivity. By both measures, kisspeptin immunoreactivity was significantly reduced in females perinatally exposed to EB, A1221 or PCB Mix compared to DMSO controls. Male AVPV kisspeptin was unaffected by any perinatal EDC treatment. ** $p < 0.001$ vs. DMSO control. PCB on the x-axis refers to the PCB Mix group.

GnRH-Fos co-expression

For GnRH co-expression of Fos, a significant effect of treatment was observed (**Fig. 5.7**; $\chi^2 = 19.07$, df = 3, $p<0.001$). Fos immunoreactivity was observed in 62% of GnRH neurons of the DMSO treated control females. Only 15% of GnRH neurons in EB treated, 6% in A1221 treated, and 8% in PCB-treated females, co-expressed Fos.

Figure 5.7: PCBs Inhibit GnRH Activation on Proestrus

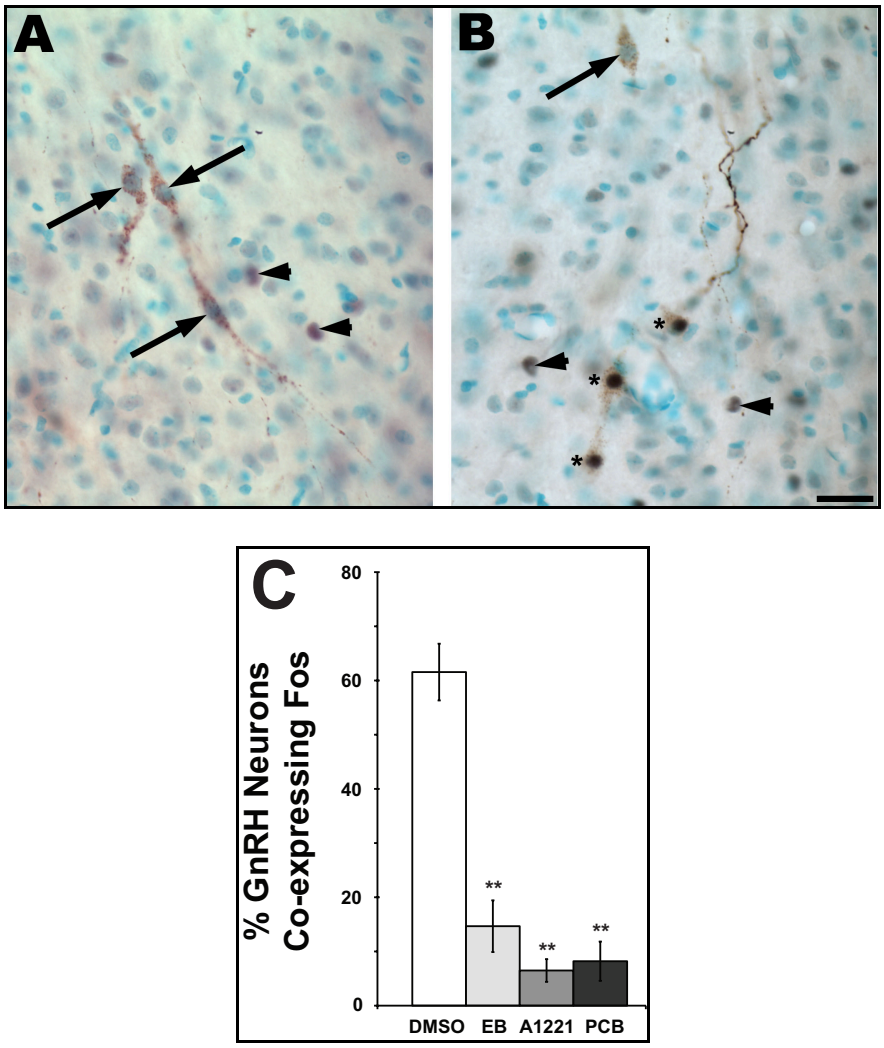

Figure 5.7: GnRH-Fos co-expression in the POA was measured in P60 female rats on the afternoon of proestrus. Examples of immunohistochemical labeling of GnRH (light brown) and Fos (dark brown nuclei) in the female OVLT are shown. Tissues are counterstained with methyl green to enable detection of cell nuclei. A) Several single-labeled GnRH neurons are seen with blue (Nissl) nuclei (arrows). Some Fos-positive nuclei (arrowheads) are seen. In this panel, none of the GnRH neurons co-expresses Fos. B) Single- and double-labeled GnRH neurons are seen. Those GnRH neurons that are immunopositive for Fos are indicated (asterisks). Single-labeled GnRH cell bodies (arrow) and single-labeled Fos nuclei (arrowhead) are also seen in that section. Scale bar A and B = 50 μm. C) The percentage of GnRH neurons of proestrous F1 females that co-expressed Fos was quantified. About 60% of GnRH neurons were double-labeled with Fos in the DMSO control group. This was significantly lower in all three neonatally EDC treated groups. Data shown are mean ± S.E.M. **p<0.001 vs. DMSO control. PCB on the x-axis refers to the PCB Mix group.

Effects of developmental PCB exposure on POA neuroendocrine gene expression

Forty-seven of the candidate neuroendocrine genes on the Rat Custom Taqman® Low Density Array (TLDA) were detectable following real-time PCR reactions (**Table 6**). Of the three potential internal controls for normalization on the TLDA card: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 18S and β-actin, our analyses showed that GAPDH did not vary across sex and treatment group. Thus POA genes were normalized to GAPDH for analyses. Of the remaining detectable genes, relative gene expression of four genes was significantly affected by developmental PCB exposure following the Bonferroni correction (**Fig. 5.8**, **Table 6**): androgen receptor, IGF-1, the N-methyl-D-aspartate (NMDA) receptor subunit NR2b, and TGFβ1. For these genes, the EDC animals had significantly decreased mRNA levels compared to vehicle in the females, but there were no significant effects in the males.

Figure 5.8: EDC Effects on P60 POA Gene Expression

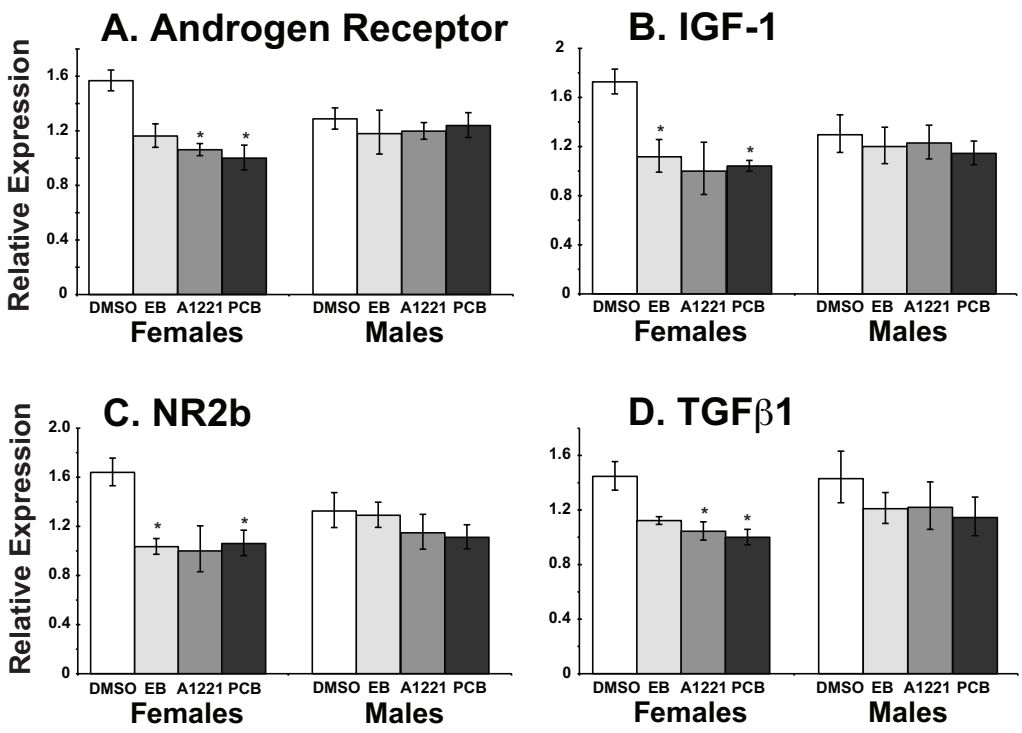

Figure 5.8: Gene expression data are shown for the four neuroendocrine genes that were significantly affected in the POA of P60 F1 male and female rats. Androgen receptor and TGFβ1 gene expression were reduced by A1221- or PCB Mix-treatment in females, compared to control. IGF-1 and NR2b gene expression were reduced in females prenatally exposed to EB or PCB Mix, compared to DMSO. Gene expression in males was unaffected by treatment. Data shown are mean ± S.E.M. N = 6 rats per group; *p<0.0001 vs. DMSO control of the same sex. PCB on the x-axis refers to the PCB Mix group.

Table 6: Low-density PCR Array Gene Expression Results for P60 Animals

		Males			Females			
Gene	**Name**	**EB**	**A1221**	**PCB**	**EB**	**A1221**	**PCB**	**Gene Expression**
Ahr	Aryl hydrocarbon receptor	--	--	--	--	--	--	
AR	Androgen receptor	--	--	--	0.002	***<0.001**	***<0.001**	**Decreased**
Arnt	Aryl hydrocarbon nuclear translocator	--	--	--	0.026	0.032	0.021	Decrease (trend)
Bdnf	Brain-derived neurotrophic factor	--	--	--	--	0.053	--	Decrease (trend)
Cyp17a1	Cytochrome P450, 17a1 (17-alpha hydroxylase)	--	--	--	--	--	0.011	Decrease (trend)
Cyp19a1	Cytochrome P450, 19a1 (aromatase)	--	--	--	--	--	--	
Cyp1b1	Cytochrome P450, 1b1	--	--	--	0.017	0.055	--	Decrease (trend)
Esr1	Estrogen receptor alpha	0.011	--	--	--	--	--	
Esr2	Estrogen receptor beta	--	--	--	--	--	--	
Gabbr1	GABA-B receptor 1	--	--	--	0.088	0.021	0.024	Decrease (trend)
Gabbr2	GABA-B receptor 2	--	--	--	--	--	--	
Gal	Galanin	--	--	--	--	--	--	
Gnrh1	Gonadotropin-releasing hormone 1	--	--	--	--	--	--	
Gnrhr	Gonadotropin-releasing hormone receptor	--	--	--	--	--	--	
Gper	G-protein coupled receptor 30	--	--	--	--	--	--	
Gria1	GluR1	--	--	--	--	--	--	
Gria2	GluR2	--	--	--	--	--	--	
Gria3	GluR3	--	--	--	--	--	--	
Grik2	Kainate 2 receptor	--	--	--	--	--	--	
Hsd17b1	Hydroxysteroid 17-beta dehydrogenase 1	--	--	--	--	--	--	
Hsd17b2	Hydroxysteroid 17-beta dehydrogenase 2	--	--	--	--	--	--	
Hsd17b3	Hydroxysteroid 17-beta dehydrogenase 3	--	--	--	--	--	--	
Hsd17b8	Hydroxysteroid 17-beta dehydrogenase 8	--	--	--	--	--	--	
Igf1	Insulin-like growth factor 1	--	--	--	***<0.001**	0.004	***<0.001**	**Decreased**

Igf1r	Insulin-like growth factor 1 receptor	--	--	--	--	--	--	
Kiss1	Kisspeptin	--	--	--	--	--	--	
Kiss1r	Kisspeptin receptor (GPR54)	--	--	--	--	--	--	
Pgrmc1	Membrane progesterone receptor	--	--	--	--	--	--	
Nmdar1	N-methyl-D-aspartate receptor (NMDAR) subunit 1 (NR1)	--	--	--	--	--	--	
Grin2a	NMDAR subunit 2a	--	--	--	--	--	--	
Grin2b	NMDAR subunit 2b	--	--	--	***<0.001**	0.005	***<0.001**	**Decreased**
Grin2c	NMDAR subunit 2c	--	--	--	--	--	--	
Grin2d	NMDAR subunit 2d	--	--	--	--	--	--	
Pdyn	Prodynorphin	--	--	--	--	--	--	
Pgr	Progesterone receptor	--	--	--	--	--	--	
Slc17a1	Vesicular glutamate transporter 1	--	--	--	--	--	--	
Slc17a6	Vesicular glutamate transporter 2	--	--	--	--	--	--	
Srd5a1	Steroid 5-alpha reductase 1	--	--	--	--	--	--	
Stat5b	Signal transducer and activator of transcription 5B	--	--	--	--	--	--	
Sts	Steroid sulfatase	--	--	--	--	--	--	
Tac2	Neurokinin B	--	--	--	--	--	--	
Tgfa	Transforming growth factor alpha	--	--	--	--	--	--	
Tgfb1	Transforming growth factor beta-1	--	--	--	0.003	***<0.001**	***<0.001**	**Decreased**
Ucp2	Uncoupling protein 2	--	--	--	--	--	--	
Vdr	Vitamin D receptor	--	--	--	--	--	--	

Table 6: Low-density PCR arrays were used to measure expression of 48 genes in whole POAs of P60 F1 female and male rats treated prenatally with EDCs. The full list of detected neuroendocrine genes is shown. Statistical results are shown for significant effects and for trends in data. Because of the Bonferroni correction, the cut-off for a significant effect was set at $p < 0.001$.

DISCUSSION

Developmental exposure to environmental EDCs can cause permanent deficits in reproductive physiology by interfering with hormone-dependent developmental processes, including brain sexual differentiation and reproductive maturation. We hypothesized that prenatal exposure to PCBs, which act in part via steroid hormone receptors, would have lasting effects on developing sexually dimorphic regions of the brain. In support of our hypothesis, we found that sub-toxic levels of PCBs administered at doses relevant for human exposures (Lackmann, 2002; Lackmann *et al*., 2004) had sex-dependent effects on reproductive neuroendocrine maturation, sexual differentiation of the hypothalamus, and neuroendocrine gene and protein expression in adulthood.

Effects on eye opening, postnatal AGD and body weight gain

A number of external markers of development were monitored in our F1 males and females. Eye opening was modestly but significantly advanced in females prenatally exposed to PCBs, but unaffected in males. As thyroid hormone signaling plays a crucial role in the onset of eye opening in rodents (Goldey *et al*., 1995), we believe that this effect may be due to effects of PCBs on the thyroid system as shown in some (Goldey *et al*., 1995; Goldey and Crofton, 1998) but not all studies (Sitarek and Gralewicz, 2009).

In this study, we also observed a slight but significant increase in postnatal body weight gain in females neonatally exposed to either EB or A1221, but not in those

exposed to the PCB Mix. Males were unaffected. Emerging evidence suggests that developmental EDCs may alter adipogenesis, energy balance and body weight (reviewed in (Hany *et al*., 1999; Bowers *et al*., 2004; Grun and Blumberg, 2005). Further work is merited to determine the mechanism for the body weight effect in our F1 females.

Other somatic and reproductive landmarks were modestly affected, including postnatal AGD in males but not females. In rodents and many other mammals, the AGD is a sexually dimorphic external marker of masculinization, with males having a longer AGD than females due largely to differences in perinatal exposures to androgens (Marois, 1968). The increase in AGD in males may seem counterintuitive, but it is consistent with a report by Kuriyama and Chahoud (Kuriyama and Chahoud, 2004) who showed that male rats exposed to the dioxin-like coplanar congener PCB118 throughout early postnatal development had hypermasculinized AGD. Other studies have shown effects of PCBs on AGD in a manner that varied by sex, congener, and timing of treatment (Wang *et al*., 2002; Lilienthal *et al*., 2006). While it is not a neuroendocrine marker *per se*, changes in postnatal AGD may be a biomarker for exposure to EDCs during early postnatal development in laboratory rodents, and is beginning to be used in human studies (Swan *et al*., 2005).

Sexually dimorphic effect of PCBs on pubertal onset

Pubertal onset was affected by perinatal PCB exposure in a sex-dependent manner, with an advancement observed in females, and a delay in males. Our results are

consistent with the results of other studies, which have found that developmental exposure to estrogenic chemicals hastens the onset of puberty in females (Delclos *et al*., 2009; Ryan *et al*., 2010) and delays puberty in males (Masutomi *et al*., 2003). The reported effects of PCBs on pubertal onset vary depending upon the dose and timing of administration, and the three-dimensional structure and half-life of the PCBs. In addition, these properties of PCBs and their metabolites also determine their interaction with steroid hormone receptors, and whether they behave as agonists or antagonists (Bonefeld-Jorgensen *et al*., 2001). For our study, two PCB mixtures were selected based on their degree of chlorination and expected half-lives. Lightly chlorinated PCBs such as those in Aroclor 1221, are believed to exert mostly estrogenic activity via either direct binding of the parent compound to estrogen receptor alpha or through interactions of its catechol or hydroxylated metabolites with ERs (Korach *et al*., 1988; Conner *et al*., 1997; Kramer *et al*., 1997; Layton *et al*., 2002; Kitamura *et al*., 2005). A1221 has a short half-life on the order of several days. In contrast, the reconstituted PCB mixture is comprised of heavily chlorinated PCB congeners that are known to accumulate and remain in biological tissues for years in humans, and have pleiotropic effects on the estrogen receptor (estrogenic or anti-estrogenic) and androgen receptor (anti-androgenic), depending upon dose (Bonefeld-Jorgensen *et al*., 2001). Our finding that the three EDCs all stimulated timing of puberty in females is probably due to the estrogenic component of these compounds.

In males, androgens play an important role in the timing of pubertal onset (Korenbrot *et al*., 1977), and anti-androgens have been shown to delay puberty (Goto *et al*., 2004). Because A1221 and one of the congeners used in our study, PCB138, has been

shown to act as an anti-androgen at low doses (Bonefeld-Jorgensen *et al*., 2001; Portigal *et al*., 2002), the delay in preputial separation observed in males prenatally exposed to the PCB Mix may be caused by an antagonistic effect on the androgen receptor. In addition, since EB also delayed puberty in males, the estrogenic properties of the three EDCs may cause an imbalance of the androgen:estrogen systems. Although not reported here, littermates of these males were utilized for a separate study, and we observed a decrease of testosterone in males at P1. Unfortunately, we did collect blood to measure testosterone at puberty, but our observation of a slight reduction in serum testosterone at P60 suggests a possible interference in the achievement of a normal secretory pattern of testosterone during pubertal maturation.

EDCs do not affect gonadal morphology but alter estrous cyclicity

EDCs such as PCBs can target all levels of the HPG axis. Previous studies have shown that reduced fertility in rats exposed to EDCs during early postnatal development is associated with abnormal gonadal morphology and abnormal estrous cyclicity in females (Adewale *et al*., 2009; Jefferson *et al*., 2009). However, very little is known about how PCB exposure during the critical neonatal window, at doses relevant for the typical human body burden, can affect gonadal development and function. Here, we found that although gonadal morphology was qualitatively unaffected by PCBs administered during the critical period, estrous cyclicity in females was disrupted. While females from the vehicle group displayed normal estrous cycles following vaginal

opening, those treated with either EB or PCBs displayed abnormal cycles characterized by 3 or more consecutive days of estrus or 4 or more days of consecutive diestrus. Because the ovarian morphology in these animals was within normal range, we suspect the abnormal estrous cycles were related to the effects of PCBs on the hypothalamic regulation of ovulation, which would be consistent with the disrupting effects of prenatal PCBs on neuroendocrine circuits described below. In addition, the male gonad showed normal seminiferous tubules and presence of sperm in all groups.

PCBs affect the hypothalamic circuits controlling reproduction in females but not males

By investigating effects of prenatal PCBs on neuroendocrine systems of males and females, we were able to test the hypothesis that females would be more vulnerable since their baseline neonatal steroid exposure is lower than that of males. In rodents, estradiol aromatized from testosterone is particularly important in brain sexual differentiation (Bakker and Brock, 2010), and PCBs can disrupt estrogenic processes. Perinatal hormone exposures of the AVPV are necessary for female rodents to develop the ability to generate the estrogen-induced GnRH/LH surge and ovulation in adulthood (Wiegand *et al*., 1978; Wiegand and Terasawa, 1982; Gu and Simerly, 1997; Le *et al*., 2001). These processes involve developmental sex differences in the number of cells in the AVPV that are immunopositive for ERα (Davis *et al*., 1996b; Orikasa and Sakuma,

2003) and kisspeptin (Clarkson and Herbison, 2006), among other sex differences (Simerly and Swanson, 1987; Simerly, 1989).

We examined the effects of prenatal PCBs on an important part of the neural circuitry controlling these processes through immunohistochemical measurements of kisspeptin immunofluorescent density and ERα immunoreactive cell numbers in the AVPV, and the activation of GnRH neurons as indicated by Fos co-localization in the POA. In the adult female F1 rats, kisspeptin- and ERα-immunoreactivity (ir) in the AVPV were significantly reduced by perinatal exposure to EB or A1221. Thus, the AVPV of these females was more male-like in its kisspeptin and ERα expression through defeminization, masculinization, or both. Moreover, GnRH-Fos co-expression on the afternoon of proestrus was substantially down-regulated in the EDC-treated female rats. The PCB Mix females, while having lower kisspeptin fiber density and GnRH-Fos co-expression, did not differ from the control group in ERα cell number, possibly due to the PCB mixture being much less estrogenic than either EB or A1221, and having more diverse molecular targets. We interpret our findings of decreased GnRH neuron activation as reflecting a disruption in the developmental programming of estrogen responsive afferents to GnRH neurons, such that AVPV ERα and kisspeptin-ir neurons do not respond robustly to estrogen positive feedback to stimulate a GnRH surge. Taken together, the observations reported here suggest that PCBs may mimic the masculinizing effects of endogenous estrogen in the developing AVPV. The effects of EB and A1221, and to a lesser extent the PCB Mix, are consistent with estrogenic exposures of the

perinatal female brain. The relative lack of effect in males is likely due to the already high exposures of the developing male brain to gonadal steroid hormones.

The results of the present study are consistent with earlier reports by our lab and others, which provide evidence that developmental exposure to PCBs and other EDCs interferes with the organization of the hypothalamus, particularly the AVPV. For instance, we have previously shown that developmental A1221 exposure down-regulates ERβ-ir in the female AVPV (Salama *et al*., 2003). Studies of developmental exposures to phytoestrogens or bisphenol A (BPA) suggest that both males and females are sensitive to the disrupting effects of these compounds on sexual differentiation of the hypothalamus. For example, developmental exposure to the phytoestrogen genistein, but not BPA (Patisaul *et al*., 2007), demasculinized male AVPV volume in adult rats, while neither of the compounds affected masculine patterns of GnRH activation or AVPV kisspeptin-ir (Patisaul *et al*., 2009). Moreover, genistein and BPA also demasculinized AVPV tyrosine hydroxylase (the rate limiting enzyme for dopamine synthesis; TH)-ir in males and defeminized the population of neurons that co-express TH and ERα in females (Patisaul *et al*., 2006). In addition, genistein, and high doses of BPA (Navarro *et al*., 2009b), defeminized AVPV kisspeptin-ir and GnRH neuron activation (Bateman and Patisaul, 2008). Collectively, these studied highlight the sensitivity of the developing hypothalamic reproductive neuroendocrine circuits to disruption by EDCs.

EDCs affect neuroendocrine gene expression in the female POA

The Taqman low-density PCR array was used to test hypotheses about a panel of neuroendocrine genes involved in hypothalamic development/function, many of which are sexually dimorphic in expression. We acknowledge that this approach is more limited in its number of molecular targets than the whole-genome microarray approach. Nevertheless, this method is powerful, as it enables us to target a subset of genes for which we had hypotheses. Furthermore, because it is PCR-based, it does not require laborious follow up as is necessitated by DNA microarrays. By this method, we identified four genes whose expression changed significantly with neonatal PCB exposure: androgen receptor (AR), IGF-1, NMDA receptor subtype NR2b, and TGFβ1. These genes were all significantly down-regulated in the POA of EDC-exposed females, but were unaffected in the males. All four of these identified genes play roles in hypothalamic development, differentiation and function, including the regulation of GnRH neurons (Gore, 2001b; Daftary and Gore, 2004; Walker *et al*., 2009). Activation of AR is critical for both defeminization and masculinization of the developing brain and body (Raskin *et al*., 2009). IGF-1 is a growth hormone that plays a crucial role in somatic growth, as well as proliferation and inhibition of apoptosis [reviewed in (Joseph D'Ercole and Ye, 2008)]. NR2b is a subunit of the ionotropic NMDA receptor, whose activation has both direct and indirect actions on GnRH neurons and the reproductive axis (Gore, 2001b; Maffucci *et al*., 2008). TGFβ1 is a cytokine protein involved in a number of

cellular functions such as cell growth, proliferation, differentiation, and apoptosis (Galbiati *et al*., 2003).

Not only have these genes been shown to regulate neuroendocrine systems, but they also interact with one another. For example, the activation of AR stimulates IGF-1 gene expression (Gentile *et al*., 2010), and interestingly, IGF-1 signaling may also stimulate AR gene expression (Fan *et al*., 2007) in a positive feedback loop. It is therefore not surprising that down-regulation of AR would be associated with decreased expression of IGF-1, and vice versa. Similarly, the promoter region of TGFβ1 has several androgen-response-elements (Qi *et al*., 2008), and may be positively or negatively regulated by AR activation depending upon the experimental model. Finally, there are numerous studies showing ineractions of NMDA receptors and IGF-1 (Le Greves *et al*., 2005; Zheng and Quirion, 2009). These gene expression data add to the story of the effects of developmental EDC exposure on reproductive neuroendocrine function, as each gene (and presumably its protein products) may directly or indirectly interact with other players in this circuitry such as kisspeptin, ERα, and GnRH whose neuroanatomy and functionality are impaired by early life endocrine disruptors.

It is informative to compare the results of protein and gene expression for the three molecules studied for both endpoints – GnRH, kisspeptin and ERα. There are clearly differences between how protein and gene expression are regulated, so some apparent discrepancies may be attributable to regulatory mechanisms such as differential effects of EDCs on gene transcription vs. post-transcriptional or post-translational levels. In addition, protein immunohistochemistry studies were done in AVPV (ERα, kisspeptin)

whereas gene expression analyses were done in the entire POA. Thus, for ERα, protein immunohistochemistry showed decreased cell numbers in the AVPV of the EB and A1221 females, whereas ERα gene expression in POA was unchanged. For kisspeptin, immunofluorescence in the AVPV was decreased in all EDC groups in females, while Kiss1 gene expression was similarly unchanged. Finally, for GnRH, gene expression was not affected by EDCs, but GnRH-Fos co-expression was decreased in the EDC females.

SUMMARY AND CONCLUSIONS

Our results show that perinatal exposure to PCBs affected the organization of the AVPV, by masculinizing or de-feminizing (or both) the female hypothalamic neurons that control reproduction. The observed effects of PCBs on AVPV development were similar to those caused by the estradiol benzoate positive control, suggesting that PCBs may act in large part through an estrogenic mechanism to target the developing neuroendocrine system. Although the link between developmental PCB exposure and human brain sexual differentiation is impossible to infer, as human sexuality is far more complicated than that of rats, and links between early life exposures and adult outcomes cannot be attributable to a single early life event. Nevertheless, PCB exposure is correlated with reduced testosterone in men (Goncharov *et al*., 2009), and reduced gonadal steroid hormones in newborns (Cao *et al*., 2008).

In addition, developmental PCB exposure affects many other neurobiological endpoints in rodents, and PCBs are correlated with behavioral and cognitive traits in

humans. In rats, behavioral development, motor function, cognitive function, and social behaviors (Cromwell *et al*., 2007; Jolous-Jamshidi *et al*., 2010). In humans, prospective longitudinal birth cohorts consistently observe impairments in executive functioning related to increased prenatal PCB exposure, including deficits in processing speed, verbal abilities, and visual recognition memory (Boucher *et al*., 2010).

In the context of environmental risk assessment, it is important to identify and characterize the neuroendocrine endpoints most sensitive to disruption, as developing humans and wildlife are likely to be exposed to PCBs and other EDCs. This research may also inform our understanding of other estrogenic EDCs in common usage in household products, food containers, plastics, and even plant products, as we are exposed to a spectrum of such compounds, both individually and in combination, at a range of dosages (vom Saal and Hughes, 2005).

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CHAPTER 6: GENERAL DISCUSSION

Recent scientific evidence has advanced our understanding of how exogenous environmental chemicals influence organisms. The field of endocrine disruption has been the focus of increasing attention from scientists and the general public as concerns mount that such chemicals may be contributing to an overall decline in human reproductive health. The structural properties of some of these compounds, known as endocrine disrupting chemicals (EDCs), allow for potential interactions with the endocrine systems of wildlife and humans. Their documented effects include actions on the reproductive, growth, metabolic, stress, and lactotrophic endocrine axes. Because the developing brain and reproductive organs are sculpted during perinatal life in part via hormonally-mediated processes, organisms are particularly vulnerable to the actions of EDCs in late gestation and early infancy.

The central focus of this chapter is the impact of environmental EDCs on the developing reproductive neuroendocrine axis. Endogenous hormones including estradiol and testosterone regulate critical periods of neuroendocrine development, the disruption of which manifests as permanent deficits in adult reproductive function. Exposure to environmental EDCs during the organization and/or activation of hypothalamic neuroendocrine circuitry can have lasting effects on reproductive physiology and sexual behavior. Importantly, exposure to EDCs is unavoidable, as a complex cocktail of such compounds is present in our daily environment. For instance, they contaminate our food and water supply, pollute our air, and are contained in products we come into contact

with on a daily basis, such as upholstery, fabrics, houseware, cookware, plastics, and even house dust. Moreover, transfer of the maternal body burden results in fetal exposure to EDCs.

EDCs are categorized into several groups, including plant phytoestrogens, industrial organohalogens, pesticides, bisphenol A, and phthalates. Our lab has been using a class of industrial organohalogen known as polychlorinated biphenyls (PCBs) as a model to investigate EDC effects on the developing neuroendocrine axis. PCBs are a family of structurally stable synthetic compounds that were globally used for industrial applications since the 1930s. Although banned in the US in 1977, PCBs continue to constitute a human health risk. Significant quantities of persistent congeners are still detectable in both the environment and in the food chain, due to bioaccumulation and biomagnification. PCBs are classified as coplanar, dioxin-like coplanar, or non-coplanar, based upon the arrangement of chlorine atoms around the biphenyl core. The structural differences between the classes of PCBs influence the binding affinity to hormone and neurotransmitter receptors, and their ability to act as an agonist or antagonist. The effects of PCBs on the developing brain are not fully understood, and a rapidly expanding literature continues to reveal previously unknown neuroendocrine targets of PCB action.

This dissertation has concentrated on understanding the cellular and molecular targets of PCBs on the developing rat neuroendocrine axis. The general hypothesis is that the PCBs tested cause changes in specific sexually dimorphic brain regions and their underlying sex-specific reproductive physiology, and neuroendocrine gene and protein expression. I also tested the hypothesis that perturbation of normal developmental

apoptosis has long-term consequences for reproductive physiology. Toward this end, the immediate and latent effects of low-dose PCB exposure during the critical period of brain organization on neuroendocrine reproductive maturation were examined. Specifically, the effect of PCBs on sexually dimorphic developmental apoptosis in the AVPV and MPN, ERα protein expression, and expression of 48 candidate neuroendocrine genes was assessed in neonatal rats at postnatal day (P) 1. Somatic and reproductive maturation was monitored until early adulthood. At P60, the volumes of the AVPV and MPN, which are shaped in part by developmental apoptosis, were measured. In addition, ERα protein expression in the AVPV and MPN, as well as kisspeptin expression in the AVPV were assessed. Neurons expressing ERα and kisspeptin send inputs to the population of hypothalamic neurons that are the central driving force for the reproductive axis: the gonadotropin-releasing hormone (GnRH) neurons. Stimulatory signals from ERα/kisspeptin neurons trigger the preovulatory GnRH surge in female rats; therefore, activation of GnRH neurons on proestrus was evaluated in females. Finally, expression of 48 candidate neuroendocrine genes was also assessed in adult rats at P60. These endpoints were selected to provide a broad assessment of how PCBs alter maturation and function of the neuroendocrine systems underlying sex-typical reproductive physiology. The results provide novel insight into the mechanisms through which exposure during late gestation to representative mixtures containing either non-coplanar or both non-coplanar and coplanar PCBs disturb neuroendocrine function in adult mammals.

In Vitro Effects of PCB Exposure on a Model Hypothalamic Cell Line

Due to their small population and sparse distribution, GnRH neurons are technically difficult to study *in vivo*. The GT1-7 cells, an immortalized mouse hypothalamic GnRH line, has provided an *in vitro* model for studying the effects of EDCs on a pure population of GnRH cells (Mellon *et al*., 1990). Aim I of this dissertation project investigated the mechanisms by which PCBs cause neurotoxicity in GT1-7 cells (Dickerson *et al*., 2009). In that study, the effects of individual PCB compounds representing different classes: PCB74 (coplanar), PCB118 (dioxin-like coplanar), PCB153 (non-coplanar), or their combination were tested. I found effects of each PCB on loss of GT1-7 cell viability, attributable to both increased necrosis and apoptosis. In general, the individual PCBs caused necrosis at the highest dosages and times of incubation, whereas apoptosis occurred at lower doses and shorter time points. Surprisingly, no additivity or synergism of the combined PCBs was observed. This may be attributable to only studying one combination that did not fully explore the range of dosages needed for such phenomena to be observed.

Cultured GT1-7 cells synthesize and release GnRH, a decapeptide that regulates vertebrate reproductive function, in a pulsatile manner. GnRH peptide levels were measured, and I demonstrate generally stimulatory effects of individual and combined PCBs at lower dosages/shorter time points, and inhibitory effects at longer time points and higher dosages. The co-administration of an ER antagonist showed that PCB-induced suppression of GnRH peptide is modulated, at least in part, by the ER. Similar results were seen for individual congeners and their combination. Collectively, the *in vitro*

results demonstrate action of specific PCBs on GnRH neurons. These results complement earlier work from our laboratory showing that the PCB mixtures A1221 and A1254 cause changes in GT1-7 cell morphology, and GnRH gene expression and peptide release (Gore *et al*., 2002).

In vivo effects

Aims II and III of this dissertation were *in vivo* studies that sought to determine whether exposure to PCBs during a critical period of brain organization would disrupt brain sexual differentiation. Two mixtures of PCBs were used, each with unique chemical properties. A1221 is a lightly chlorinated mixture of estrogenic coplanar and non-coplanar PCBs with a short half-life. The reconstituted mixture comprised of the more heavily chlorinated non-coplanar congeners PCB138, PCB153, and PCB180, has a long half-life. The individual congeners contained in this mixture have been reported to be estrogenic, anti-estrogenic or anti-androgenic (Bonefeld-Jorgensen *et al*., 2001). I found that exposure of female rats to these PCBs at environmentally relevant doses during late gestation had both immediate (P1) as well as long-lasting effects on neuroendocrine reproductive physiology observed during the pubertal transition and early adulthood (P60).

In female rodents it is believed that the attainment of adult reproductive capacity requires the organization of a hypothalamic neural network of GnRH cells, together with their regulatory inputs, that facilitate steroid hormone feedback effects on pulsatile GnRH

release, and the preovulatory GnRH/LH surge in females. The morphological and neurochemical organization of this steroid-responsive neural network begins during late gestation and infancy, is regulated by hormones, and can be manipulated by gonadectomy or administration of exogenous hormones (Piacsek and Meites, 1966; Gross, 1980; Jean-Faucher *et al*., 1985). In male rats, androgens released by the fetal/neonatal testes, and their subsequent aromatization to estradiol within the nervous system, masculinizes and defeminizes the male brain, in part via stimulation or inhibition of programmed cell death (apoptosis). Conversely, the female rat brain is exposed to levels of estradiol several orders of magnitude lower than the male brain, and is demasculinized and feminized during development.

My experiments show that A1221 or EB increased the extent of AVPV apoptosis in P1 females, but not males. However, the reconstituted PCB mixture had no effect on developmental apoptosis. The discrepancy between the effects of these two PCB mixtures on AVPV apoptosis may be related to differences in their interaction with the estrogen receptor, although the current experimental design did not allow for that determination.

I found that the ERα-ir cell number was increased in the P1 MPN by EB treatment in both males and females, while both PCB mixtures had no effect. Thus the PCB mixtures studied do not have the same effects as an exogenous estrogen for all endpoints. Finally, I found altered gene expression of nine factors involved in neuronal migration and anatomical establishment of hypothalamic brain nuclei in P1 pups of both sexes, including brain-derived neurotrophic factor (BDNF), GABAB receptors 1 and 2, IGF-1, kisspeptin receptor (GPR54), NMDA receptor subtypes NR2b and NR2c,

prodynorphin, and TGFα. Specifically, in females, EB and A1221 increased expression of both GABAB receptor 2 and NMDA2b. Although there was a trend for PCB-induced gene expression changes by the reconstituted mixture, these did not reach statistical significance following Bonferroni correction. In males, EB treatment reduced gene expression of BDNF and KISSR, while increasing NMDA2c. A1221 increased IGF-1. The reconstituted PCB mix increased gene expression of both GABAB receptor 2 and TGFα, while decreasing KISSR. As a whole, these results show that a network of genes and proteins is altered in the hypothalamus as early as the day after birth, with results dependent upon the specific EDC administered.

The initiating event in the pubertal transition to adult reproductive capacity is activation of GnRH release from the hypothalamus, which stimulates the release of gonadotropins from the pituitary, which, in turn, activates the gonads. Development of secondary sex characteristics results from increased levels of circulating sex steroid hormones. Sex steroids activate the sex-specific brain morphology and neurochemistry that had previously been organized during early postnatal life. Because puberty is sensitive to endogenous hormones, it is not surprising that this postnatal developmental transition is also sensitive to EDCs. Moreover, puberty itself may be associated with further organizational or reorganizational changes in the nervous system and is beginning to be considered a second critical period for brain sexual differentiation (Sisk and Foster, 2004). In my dissertation research, PCB exposures during early life hastened puberty in females, and delayed it in males.

A key regulatory region in the control of the hypothalamic GnRH neuronal network is the AVPV, a preoptic brain region that expresses nuclear hormone receptors such as ERα (Simerly *et al*., 1990), ERβ (Orikasa *et al*., 2002), and PR (Quadros *et al*., 2002), as well as other neuromodulators such as kisspeptin (Clarkson and Herbison, 2006) in a sexually dimorphic manner. The adult female rat AVPV is larger than in male rats (Sumida *et al*., 1993), resulting from a sex difference in developmental apoptosis in this region, which is normally greater in males than females. A subset of neurons within the AVPV directly innervates GnRH neurons (Hahn and Coen, 2006; Polston and Simerly, 2006), enabling the positive feedback effects of steroids upon the preovulatory GnRH/LH surge (Wiegand *et al*., 1978; Wiegand and Terasawa, 1982; Gu and Simerly, 1997; Le *et al*., 2001), a phenomenon that is latent in males. This population of neurons modulates GnRH release in part via expression of ERα (Couse *et al*., 2003; Wintermantel *et al*., 2006) and kisspeptin (Clarkson *et al*., 2008). I examined the effects of prenatal PCBs on this neural circuitry in adults. The results of this study suggest that these processes may be impaired by the prenatal masculinization or defeminization (or both) of the female's AVPV.

In female rodents, rising levels of estradiol released from ovarian granulosa cells during the follicular phase of the estrous cycle trigger a shift from negative to positive steroid feedback onto the hypothalamus. Estradiol then stimulates a surge in GnRH release from neuroterminals in the median eminence in part via stimulatory inputs from a sexually dimorphic population of neurons in the AVPV that co-express ERα and kisspeptin (Franceschini *et al*., 2006; Adachi *et al*., 2007; Clarkson *et al*., 2008). The

response of GnRH neurons to estradiol positive feedback can be detected through their activation, as evident by Fos co-expression. I found that EB- and A1221-treatment reduced AVPV volume in adult females, a result consistent with the increased AVPV cell loss measured at P1. Interestingly, serum levels of estradiol and progesterone in F1 offspring at adulthood were not affected by their prenatal PCB exposure, suggesting that steroid hypothalamic feedback is potentially unaffected. Moreover, ovarian samples from all treatment groups showed corpora lutea, a reliable indicator that ovulation occurred in all females. Nevertheless, the GnRH neurons of the prenatally exposed females did not respond normally to ovarian steroid feedback, implicating a central mechanism for PCB effects on female reproductive physiology. That is, on the afternoon of proestrus, the expression of both ERα and kisspeptin was reduced by prenatal EB or PCB exposure in the adult female AVPV. The co-expression of Fos in GnRH neurons of these animals was concomitantly and dramatically reduced. The finding of disrupted estrous cyclicity in the context of reduced AVPV ERα-ir, reduced AVPV kisspeptin-ir and an inhibition of GnRH activation on the afternoon of proestrus suggests that prenatal PCBs interrupt developmental programming of the hypothalamic circuits controlling the preovulatory GnRH/LH surge in adult females.

In adult males AVPV ERα-ir cell number and kisspeptin-ir were not affected by treatment. The reconstituted PCB mix increased male AVPV volume, while the other treatments had no effect. This relative lack of effect in males may be related to the already high exposure of their developing brain to gonadal steroid hormones.

In addition to the changes in protein expression, I also found that gene expression of the following four neuroendocrine genes was affected by developmental EDC exposure: androgen receptor (AR), IGF-1, the N-methyl-D-aspartate (NMDA) receptor subunit NR2b, and TGFβ1. In females, both A1221 and the reconstituted PCB mix decreased AR and TGFβ1 mRNA levels compared to vehicle, while EB and A1221 decreased expression of IGF-1 and NR2b. There were no significant effects in the males.

Based on these data, I propose a model for PCB disruption of hypothalamic programming, below.

A Comprehensive Model for PCB Disruption of Hypothalamic Developmental Programming

Taken together, these data suggest a model whereby PCBs disrupt developmental programming of the female hypothalamus, a process that begins in the neonatal animal, continues throughout pubertal development, and is manifested as neuroendocrine reproductive deficits in early adulthood. Therefore, the proposed model begins with PCB disruption of hypothalamic organization through interference with the anatomical establishment of AVPV neural circuitry via aberrant neuronal cell death and migration (**Fig. 6.1**). In the developing hypothalamus, PCBs can interfere with proper anatomical and neurochemical establishment of the AVPV via direct or indirect interactions with estrogen receptors, stimulation of aberrant apoptosis, and/or interference with expression of neurotrophic and migration factors.

Figure 6.1: Perinatal Programming of the AVPV

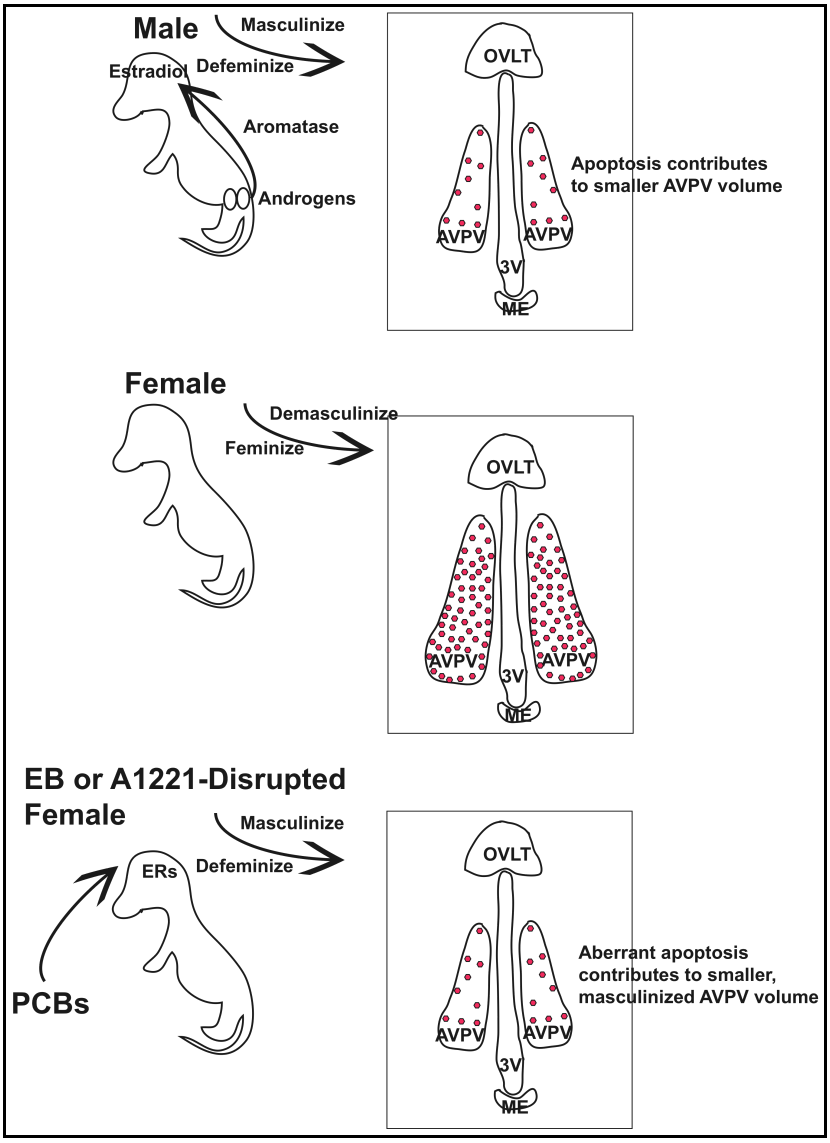

Figure 6.1: Model for the normal organizational effect of sex steroid hormones on the developing hypothalamus. In male rats, estradiol, aromatized in the brain from androgens secreted by the perinatal testes defeminizes (reduces) the number of AVPV kisspeptin and ERα neurons. In contrast, female brains are exposed to estradiol and androgens at several orders of magnitude lower than males during the perinatal developmental period. Therefore, the female brain is feminized and demasculinized, developing an AVPV with much higher numbers of kisspeptin and ERα neurons. In the neonatal EB- or A1221-disrupted female, PCBs increase developmental apoptosis, thereby defeminizing and masculinizing this system. Thus, the AVPV develops in a male-typical fashion, with a smaller volume, and reduced numbers of neurons that express kisspeptin and ERα.

The next step in the proposed model is PCB disruption in the activation of hypothalamic GnRH release at puberty (**Figure 6.2**).

Figure 6.2: PCB Disruption of Pubertal Activation of GnRH Release

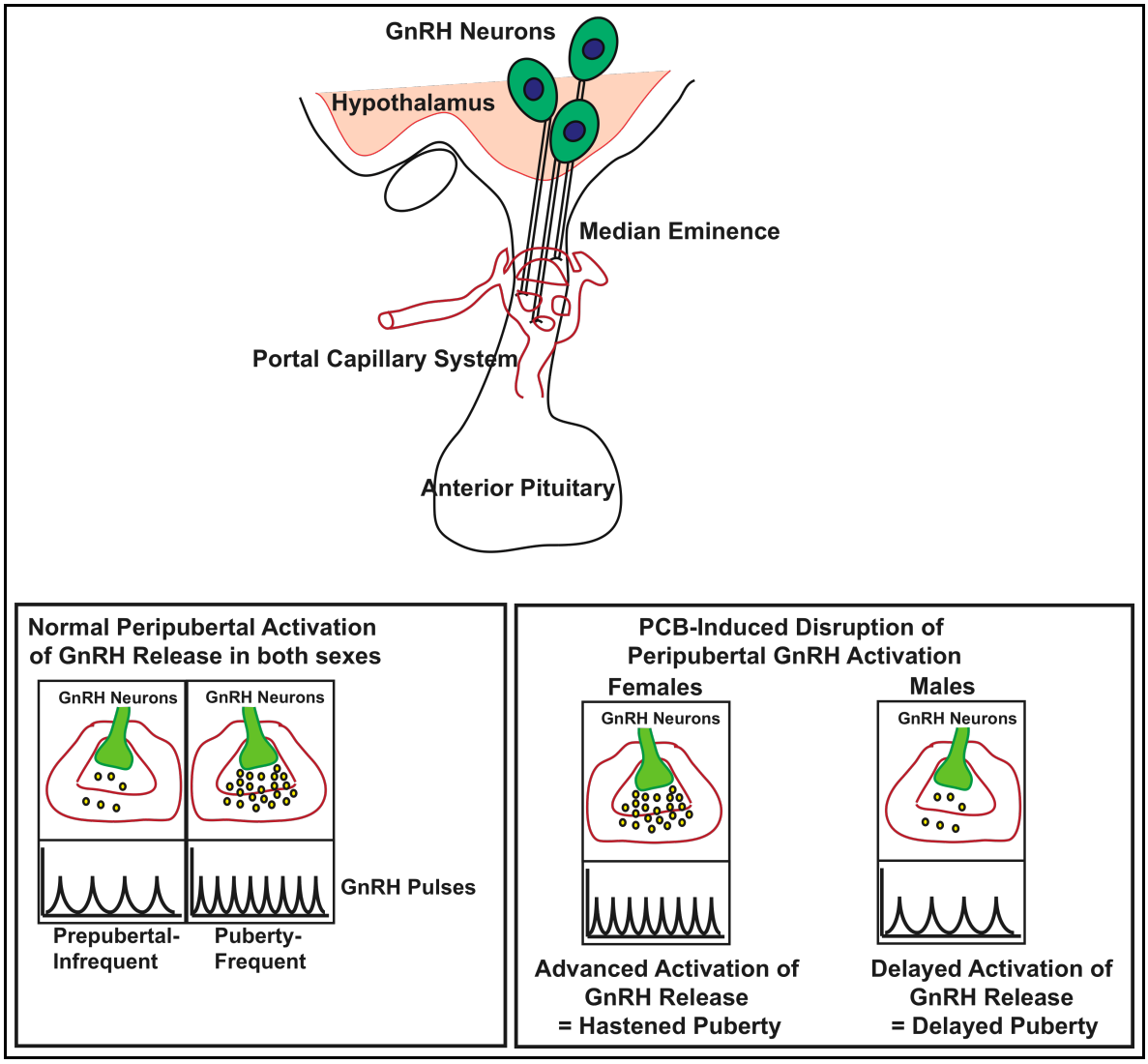

Figure 6.2: Model for PCB-induced disruption of normal activation of GnRH release at puberty. GnRH nerve terminals in the median eminence are shown in green; small circles represent secretory vesicles that are released from the GnRH neurons in a pulsatile pattern of about once every 30-90 minutes, depending upon age and sex.

The final step in the proposed model is a disruption in the integration of steroid feedback with stimulatory signals from AVPV ERα/Kisspeptin neurons onto GnRH neurons, leading to diminished or delayed activation of the GnRH/LH surge on the afternoon of proestrus (**Figure 6.3**). The male hypothalamus also undergoes maturation at puberty, although the response to positive steroid hormone feedback is latent in males. The lack of treatment effect upon male AVPV ERα and Kisspeptin protein expression may be related to the already high exposure of their developing brain to gonadal steroid hormones.

Figure 6.3: PCB Disruption of Neural Circuitry Controlling GnRH Surge

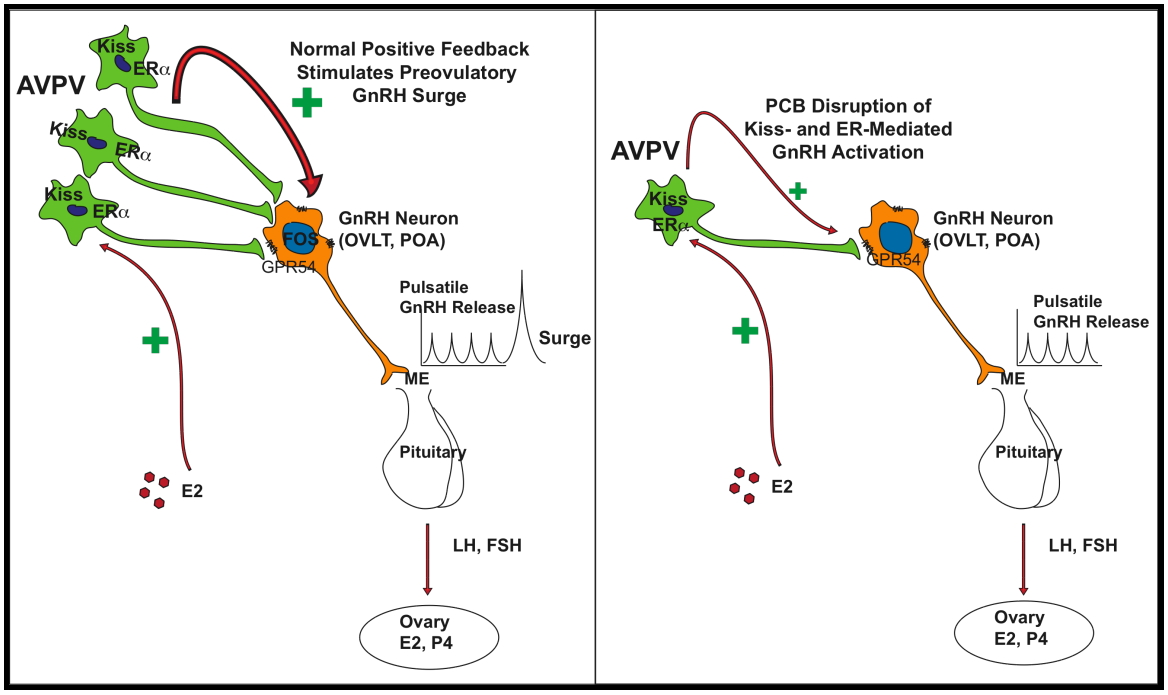

Figure 6.3: Prenatal PCBs disrupt the neural circuitry regulating the preovulatory GnRH surge. In the female AVPV, neurons expressing kisspeptin and ERα extend projections to GnRH neurons in the organum vasculosum of the lamina terminalis (OVLT) and preoptic area (POA). In response to the positive feedback triggered by rising levels of estradiol (E2) released by the ovary during the follicular phase of the estrous cycle, AVPV kisspeptin/ERα neurons send stimulatory inputs to GnRH neurons, which express GPR54, the kisspeptin receptor. Activation of GnRH neurons is evident by co-expression of FOS, and is followed by the preovulatory GnRH/LH surge. In females prenatally exposed to estradiol benzoate or PCBs, positive feedback from reduced numbers of kisspeptin/ERα neurons is not completely abolished, but is insufficient to activate GnRH neurons on the afternoon of proestrus.

CONCLUSIONS

In this series of experiments, developing male and female rats were exposed to ecologically relevant low doses of PCBs during the period of brain sexual differentiation. The exposed F1 generation displayed no gross morphological abnormalities, and litter size and sex ratio were not affected. However, treated males and females exhibited disruptions in reproductive neuroendocrine development, with females being more susceptible to this type of impairment. Developmental profiles of apoptosis, gene and protein expression in the POA revealed effects of PCBs as early as the day after birth, while results from the pubertal and early adult endpoints highlighted the latent and long-lasting impact of PCB action on the developing hypothalamus. Collectively, the *in vivo* results point to a comprehensive model for PCB disruption of developmental AVPV programming in the female rat, whereby increased AVPV apoptosis and altered POA gene expression of neurotrophic and cell migration factors lead to defeminization/ masculinization of the AVPV, followed by early activation of GnRH release in the peripubertal period, and finally manifesting as a dysregulation of steroid hormone positive feedback integration with AVPV stimulation of GnRH activation at proestrus. These data contribute important new information to the growing body of literature whose aim is to better understand the fetal basis of adult diseases.

The results of this dissertation research have relevance for human public health and disease, as increased trends for reproductive decline (Chandra *et al*., 2005) have led researchers to hypothesize that environmental endocrine disruptors are adversely

affecting human health and reproduction. Indeed, the human evidence for endocrine disruption by environmental EDCs is mounting [reviewed in (Diamanti-Kandarakis *et al*., 2009)], and highlights the importance of linking basic mechanistic research with epidemiological observations of obesity, reproductive cancers, sperm counts, diabetes, and precocious puberty in humans. As humans are rarely exposed to a single EDC, and there are limited data on the interactions between compounds that comprise the typical environmental mixture of different classes of EDCs, future research must clarify the effects of simultaneous exposure to compounds which may have additive, synergistic, or antagonistic effects (Kortenkamp, 2008).

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